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A Promising Novel Method Targeting Prosthetic Joint Infections

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Abstract

Approximately 1-2% of patients have complications due to bacterial infection. *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* are the three bacteria responsible for approximately 75% of biofilm-related infections. These three microorganisms can adhere to stainless steel and orthopedic screws. Biofilms are produced by an accumulation of bacteria which can attach to a surface, accumulate and form an extracellular polymeric substance.

Green tea extracts from plant *Camellia sinensis* have long been studied for their antimicrobial effect on gram-positive and gram-negative bacteria. Polyphenols found in green tea, specifically epigallocatechin-gallate (EGCG), are the most important component in targeting biofilm. However, this compound can be readily oxidized and is therefore unstable. Previous studies have shown that the modified lipophilic compound, epigallocatechin-gallate-stearate (EGCG-S), can inhibit biofilm formation and decrease cell viability.

The purpose of this study was to evaluate if EGCG-S could play a role in targeting prosthetic joint infection caused by *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. Major goals of this study were to determine if EGCG-S could enhance the current treatment method and inhibit biofilm in addition to two virulent factors. The minimum time frame was also determined for treatment to be most effective. EGCG-S was shown to have some inhibitory effects on colony forming units, the most effective time for treatment was observed starting at five minutes. The tea polyphenol was also able to inhibit biofilm and protease and elastase when the three

bacteria were in combination. EGCG-S shows potential for combating acute and chronic joint arthroplasty infections.

MONTCLAIR STATE UNIVERSITY

A Promising Novel Method Targeting Prosthetic Joint Infections

by

Shanice Otieno

A Master's Thesis Submitted to the Faculty of

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In Partial Fulfillment of the Requirements

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Thesis Committee:

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A PROMISING NOVEL METHOD

TARGETING PROSTHETIC JOINT INFECTIONS

A THESIS

Submitted in partial fulfillment of the requirements

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Montclair State University

Montclair, NJ

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Introduction

The wear and tear of everyday activities is extremely burdensome on joints. Osteoarthritis, a degenerative joint disease caused by wearing and tearing of the breakdown of cartilage covering the ends of bones in joints is due to chronic inflammation. In this disease, tissues such as cartilage start to erode away, causing an immense amount of pain, discomfort and overall debilitation (Song et al., 2013). With the current population's life expectancy on the rise, osteoarthritis has become the fourth most common cause of hospitalization in 2009. The end result of osteoarthritis after failed conservative methods is usually joint replacement surgery (Song et al., 2013) Thus, there has been an increase in orthopedic surgery for prosthetic joint implantations.

Advancements in joint implantations have allowed individuals to regain their mobility and overall physical function. In 2005 there were 572,000 and 3.48 million cases of hip and knee replacement surgeries, respectively. It was estimated then that by 2030 the need for primary total hip arthroplasties and total knee arthroplasties would be projected to grow by 174% and 673% (Kurtz et al., 2007). Due to increased knowledge in healthcare and technological innovations, the amount of prosthetic joint implantations has tremendously increased in orthopedic medicine. In 2010 there were over one million total knee and hip arthroplasties. Additionally, Tande and Patel (2014), suggest that the total number or surgical interventions will increase to 5 million by 2030, more than was originally predicted.

As the incidence rises there are minimal risks; however, one of the side-effects is bacterial infection due to aseptic or septic failure (Kasch et al., 2016). Infections can be

classified as early, delayed, or late onset of symptoms after implantation; early being within three months of a patient's last surgery, delayed occurring after three months but before two years post-surgery, and late occurring past a two-year time period (Tande & Patel, 2014). Infections can also be classified according to their route of entry such as perioperative, hematogenous, and contiguous. Perioperative suggests that infection is due to the inoculation of the microorganisms during surgery or immediately after. Patients who are immunosuppressed tend to be at higher risk for perioperative infections. Haemateogenous infection means the infection has spread through blood or lymph. Contiguous infection is one that spreads from an adjacent focus of the infection, such as a penetrating trauma, osteomyelitis, skin or soft tissue lesions (Trampuz & Zimmerli, 2005).

Causative agents of these infections are usually gram-positive staphylococcus and streptococcus (Rodríguez-Merchán & Liddle, 2018). Gram-negative bacteria also constitute 10-23% of infectious episodes that are acute (Rodríguez-Pardo et al., 2014). *Staphylococcus aureus* is a microorganism that is largely responsible for prosthetic joint infections, followed by coagulase-negative staphylococci, such as *Staphylococcus epidermidis* (Tandel & Patel, 2014). Aerobic gram-negative bacteria such as *Pseudomonas aeruginosa* is another dominant bacterium responsible for infections (Stover et al., 2000). Humans are natural reservoirs for these three bacteria. These bacteria reside in patient's blood, nasal passages, and skin. When combined, these polymicrobial prosthetic join infections can occur in up to 35% of early onset infections (Tande & Patel, 2014).

Staphylococcus aureus, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* are the three predominant bacteria responsible for approximately 75% of biofilm-related infections in prosthesis. These three opportunistic microorganisms have the ability to adhere to metals such as cobalt-chromium, titanium, polyethylene, and polymethylmethacrylate (PMMA) cement which are commonly used in implants, and orthopedic screws creating a biofilm (Song et al., 2013; Nana et al., 2016). Biofilm formation is due to a unique interaction of unicellular organisms that are encompassed in an extracellular matrix of polysaccharides, proteins, and nucleic acids (Fey & Olson., 2010). Biofilm formation is broken down into four steps: adherence, accumulation, maturation and detachment (Fey & Olson, 2010). The clusters of microorganisms living closely together in this matrix depletes metabolic substances and increases products, allowing the accumulation to cause the microbes to enter a slow or non-growing stage. Thus, the microorganisms are up to 1,000 times more resistant to antimicrobial treatments than if they were freely living on their own. Their complex structure allows for interstitial water channels in which nutrients can circulate, forming a system similar to a circulatory system which then allows for the release of cell to cell signaling molecules known as quorum sensing. Quorum sensing allows bacteria in a population to respond to changes in gene expression, thus allowing the biofilm to differentiate (Trampuz & Zimmerli., 2005). Additionally, quorum sensing can cause transcriptional changes, which can lead to expression of virulence factors and antibiotic resistance (Nana et al.,2016).

Gram-positive and negative bacteria use different types of quorum sensing systems. One system gram-positive bacteria use is through peptides called autoinducing peptides. Gram negative bacteria communicate using extracellular signaling molecules

called autoinducers (Rutherford & Bassler., 2012). Common virulence factors produced by these bacteria are protease and elastase, which help with the quorum sensing to form biofilms. Proteases are produced by wide variety of microbes, which are not only resistant to human plasma protease inhibitors but can inactivate these inhibitors thus accelerating the infection process (Maeda,1996). Elastase, an enzyme in the class of proteases, breaks down elastin and elastic fibers. Neutrophil elastase has been shown to impair host defense against infection by degrading phagocyte surface receptors and opsonins produced by bacteria (Hiemstra, 2002). Proteases are important in tissue damage caused by *P. aeruginosa* infection. *P. aeruginosa* secretes alkaline protease and two elastases (A and B) have been characterized as exoenzymes and virulence factors (Engel et al. 1998). Pavlovskis and Wretlind (1979) found that protease enhances the virulence of *P. aeruginosa* and is a contributing factor to the invasiveness of the pathogen. *S. epidermidis* and *S. aureus,* have a similar core genome, although *S. aureus* is usually associated as more virulent than *S. epidermidis*. *S. epidermidis,* encodes fewer known and putative virulence factors and pathogenicity islands in comparison to *S. aureus*. Some of the virulence factors produced by *S. epidermidis* are serine protease, cysteine protease, elastase, and Clp protease (Fey & Olson, 2010). Therefore, one mechanism to inhibit biofilm formation would be to inhibit these virulence factors in order to prevent cell attachment or aggregation on the device.

S. aureus produces various enzymes that clot plasma and coat the bacterial cell, which helps evade the immune system response by preventing phagocytosis. One enzyme that does this is coagulase. Another enzyme produced by *S. aureus* is hyaluronidase, which breaks down hyaluronic acid, and helps spread the bacteria. *S. aureus* also

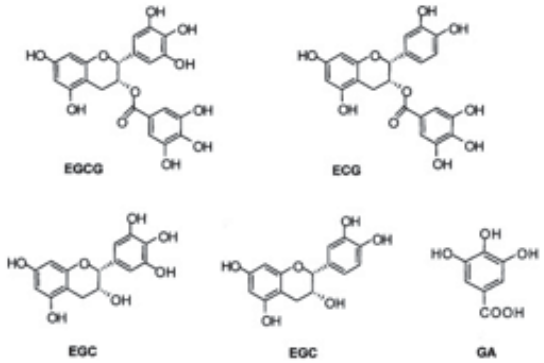
produced exotoxins that can promote its virulence. Costerton, Montanaro, and Ariola (2005) suggest that biofilm formation in staphylococcus bacteria is a polymer β-1,6-linked N-acetylglucosamine (PIA), whose synthesis is mediated by the *ica* operon. Ruiz de los Mozos (2013) targeted a long 3′-untranslated region of *icaR*, which codes for the repressor of the main exopolysaccharide compound of the *S. aureus* biofilm matrix and found that deletion or substitution in a specific area resulted in inhibition of biofilm formations. Other proteins that have been associated with biofilm formation in staphylococcal bacteria are accumulation-associated protein (AAP), the clumping factor A (ClfA), the staphylococcal surface protein (SSP1) and the biofilm-associated protein (Bap) (Gotz., 2002).

Pseudomonas aeruginosa is also considered a successful opportunistic pathogen because it produced a myriad of virulence factors such as exoproteases, siderophores, exotoxins, and lipases. There are two quorum sensing systems in *P. aeruginosa*: LasR-I and RhlR-I, with an additional LuxR homolog that is adjacent to a cluster of quorum-sensing-controlled (*qsc*) genes. The target genes for these systems are *lasB, toxA, rhIR, and lasl* (Parsek & Greenberg, 2000).

Although, 1% of prosthesis joint implantation become infected, this complication is associated with higher morbidity and a substantial economic hardship. Treatment is usually a two- stage procedure in which there is first the removal of the foreign material, such as the implant and bone cement, followed by a course of antimicrobial treatment. This conservative approach has a success rate for staphylococcal infections ranging from 55% to over 75% (Rodríguez-Pardo et al. 2014). Other surgical interventions include debridement with retention, in which the surgical area is washed out and the implantation

is kept in. The one stage direct exchange is the removal and implantation of a new prosthesis. Lastly, the two-stage surgical intervention is removal of the prosthesis and implantation of a new one, two to eight weeks later. Worst-case scenario would be permanent removal of the device, which is usually done for patients with high risk of reinfection such as those that are severely immunocompromised. Antimicrobial therapy is suggested for a 3-month duration for hip prostheses and 6 months for knee. The most used therapy is rifampicin for staphylococcal implant infections (Trampuz & Zimmerli, 2005).

Currently, the best way to resolve the issue of the burdensome biofilm is the removal of the implanted device, a solution that is extremely expensive. Some new novel approaches have been studied such as the use of green tea polyphenols. Green tea from plant *Camellia sinensis* has long been studied for its antimicrobial effect in gram positive and gram-negative bacteria (Reygaert, 2014). Green tea is a non-fermented tea, which contains more catechins than other processed tea. Tea polyphenols are strong antioxidants both *in vitro* and *in vivo*. Polyphenols, specifically flavonoids such as catechins are present in green tea. The four major catechins are epigallocatechin-3-gallate (EGCG), that represents approximately 59% of the total of catechins, epigallocatechin (EGC) (19% approximately), epicatechin-3-gallate (ECG) (13.6% approximately), and epicatechin (EC) (6.4% approximately) (Cabrera, Artacho & Giménez, 2006).



Structure 1. Molecules of green tea catechins
(Cabrera, Artacho, & Giménez 2006)

It has been reported that the antimicrobial properties of EGCG are mediated through its high affinity but nonspecific binding to viral surface proteins (Hsu,2015). EGCG is a potential agent in treating bacterial infections because it is non-toxic. However, when placed in an aqueous environment, it is readily oxidized and loses its stability (Ping et al., 2003). Thus, it has been proposed that fatty acid modified polyphenols, as through lipid esterification, could be effective. These enzymatic and chemically altered compounds are referred to as lipid soluble tea polyphenols and are more stable (Ali et al., 2017).

While many studies have shown that EGCG has effective antimicrobial effects, there have not been sufficient studies in the host evading mechanisms of the bacteria due to EGCG treatment. Tran et al (2014) completed a promising study in which they looked at the minimum inhibitory concentration of EGCG that could interfere with quorum sensing regulating factors such as protease, elastase, and pyocyanin produced by *P. aeruginosa* and *P.fluorescens*. They found that as the concentration of EGCG increases, there was a decrease in biofilm production in the two bacteria (Tran et al., 2014). The Minimum Inhibitory Concentration for each organism was found to be 8 mM, which is approximately 3.6 mg/mL of EGCG. Polyphenols found in green tea, specifically

epigallocatechin-gallate, are an important component in targeting biofilm. This study seeks to expand on the current treatment method currently used by Dr. Alvin Ong and other orthopedic surgeons at AlantiCare Regional Medical Center and Thomas Jefferson University Hospital. A further elaboration on the study of Tran et al (2014), will also be performed to evaluate the inhibitory effect of virulence factors in biofilm formation using epigallocatechin-gallate-stearate (EGCG-S), a green tea polyphenol. It was hypothesized that EGCG-S at a concentration of 250 μg/ml would have an enhancing effect on the current treatment method and an inhibitory effect on protease and elastase virulent factors in biofilm production of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa,* and a combination of all three.

Objectives

1. Evaluate current prosthetic joint infection treatment method with EGCG-S.
2. Determine minimum synergistic time frame for antibiotics and EGCG-S.
3. Determine synergistic effect of EGCG-S on biofilm and two virulent factors.

Materials and Methods

1. Culturing Bacteria

Three strains of bacteria were used: *Staphylococcus aureus* (*S. aureus,)* *Staphylococcus epidermidis* (*S.epidermidis*) and *Pseudomonas aeruginosa (P.aeruginosa*). Each strain was kept and maintained on a tryptic soy agar (TSA) plate. The plates were stored in a refrigerator at 4^{o}C and re-grown on new tryptic soy agar plates every two weeks. Overnight cultures were made from the stock plates, by obtaining a small amount of the bacteria (aseptically) with a cotton swab or inoculation loop. After the sample was obtained, it was put into a tube with tryptic soy broth (TSB). The samples with broth and bacteria were then labeled with the initials of the organism, date and the experimenter's name. Samples were then placed into a 250-rpm shaking incubator at 37^{o}C. The samples were removed from the incubator the next morning. Before starting the experiment, the overnight cultures were checked for purity by preforming a simple or gram stain.

2. Media Preparation

a. Tryptic Soy broth (TSB) preparation

Thirty grams of powder from Difco^{TM} was mixed with 1L of deionized (DI) water in a 2L Pyrex flask. The flask was labeled with tape for indication of the contents inside and the experimenter's name. Once mixture was mixed thoroughly (powder fully dissolved), it was autoclaved on the liquid cycle for 30 minutes at 121^{o}C. When the autoclave process was complete the flask was allowed to cool and later placed in a 4 ^{o}C cold room for storage.

b. Tryptic Soy (TSA) and Muller Hinton Agar (MHA) preparation

Forty grams of tryptic soy powder from Difco™ was dissolved in 2L flask with 1L of deionized water. The flask was labeled and autoclaved at 121°C for 30 minutes. The flask was allowed to cool to about 55-60 °C. Once cooled the liquid was poured into sterile plates aseptically. The plates were kept under the hood, until they solidified. Once solidified, plastic sleeves were used to store the plates for future use. The sleeves were labeled with the name of the agar and placed in the cold room.

MHA plates were prepared for Disk diffusion studies by combining 1L of deionized water with 38g of powder from Difco™. The solution was mixed and autoclaved at 121 °C for 30 minutes. After cooling, the media was poured into sterile plates. Once the agar solidified on the plates, they were put into sleeves, labeled and stored in the cold room for future experiments.

3. Staining Techniques

a. Simple stain

A simple stain was performed on overnight cultures before the start of each experiment to check for purity. A sterile loop was used to obtain a small sample. The sample was then placed on a sterile microscope slide, heat fixed and stained with methylene blue. A coverslip was placed on top of the sample and bibulous was used to blot dry the slide. The slide was viewed under the oil immersion objective (1,000X) with a compound microscope to observe cell morphologies (Lee et al., 2015).

b. Gram Stain

Gram stain was carried out to check and confirm the gram status and to determine the morphology of the microorganisms to be used in the experiment. A sample was taken

from TSA or TSB. If TSA was used a drop of DI water had to be placed on the slide. The bacteria from the agar was then mixed in. Once a sample was placed on the slide it was heat fixed. Crystal violet was then applied for 20 seconds and rinsed with DI water for 2 seconds. Gram's iodine was added for 1 minute and washed off with Gram's decolorizer for 10-20 seconds. The decolorizer was then rinsed with DI water for 2 seconds and safranin was applied for 1 minute. The safranin was rinsed with DI water and the slide was blot dried with bibulous paper. Observation of the slide was done under a compound microscope at 1,000X (Lee et al., 2015).

4. Preparation of treatments

a. Tea Polyphenols

EGCG-S was purchased from Camellix LLC, Augusta, GA. A stock of 10X 250 µg/mL and 10X 500 µg/mL were prepared. The compounds were dissolved in 200 proof ethanol. The stocks were stored in a -20°C freezer.

b. Antibiotics

Polymyxin B and Bacitracin, the two antibiotics currently used to treat joint infection at Atlantic Regional Medical Center were prepared. The powders were purchased from Sigma Aldrich. Masses of 0.00198 g of Polymyxin B and 0.00225 g of Bacitracin, respectively, were weighed out and each dissolved in 10 ml of DI water (100X). Commercial polymyxin B (300 µg/mL) disks were purchased from Carolina Biological. The antibiotics were stored in a 4°C fridge.

c. Wash (W)

The wash that is currently used for irrigation was prepared by combining 10 ml of 0.9 % saline with 1.57 ml of 0.25% sodium hypochlorite and 0.3 ml povidone iodine (Dynarex).

5. The Effects of Current treatment and ECGG-S on inhibition of PJI

a. Kirby-Bauer Disk Diffusion Test

Bacteria were swabbed throughout an MHA plate. Sterile 6 mm diameter BBL ™ disks were then soaked in 1mL of the appropriate surgical wash solution for 1 minute (saline, sodium hypochlorite and povidone iodine (Wash, W)). A 12 well plate was used to soak the disks, the disks were removed from solution after 1 minute and allowed to slightly air dry in an empty well. The disks were then placed aseptically on a MHA plate.

Disks for antibiotics and tea were also prepared by soaking sterile 6 mm BBL ™ disks in solution for 1 minute and allowing it to air dry for the same time frame. A concentration of 100X antibiotics was obtained from the original prepared stock. Concentration of 10X antibiotics was prepared by obtaining 100 µl of stock antibiotics and adding it to 900 µl of DI water. Concentration of 1X was prepared by obtaining 100 µl of 10X antibiotics and adding it to 900 µl of DI water. One ml of 250 µg /ml and 500 µg /ml of EGCG-S were taken from the stock. BPT (bacitracin, polymyxin and tea) solution was prepared by combining 350 µl of DI H_2O, 50 µl of 10X bacitracin, 50 µl of 10X polymyxin B and 50 µl of 250 µg/ml EGCG-S. Commercial Polymyxin B 300 µg (PC, PB300) was purchased from Carolina Biological.

b. Determining Optimal Time for Treatments

Overnight cultures for *S.aureus* and *S. epidermidis* were treated for 30 seconds, 1 minute, 5 minutes, 30 minutes and 1 hour. 100 µl of the overnight culture was removed from a 15 ml tube and placed into an Eppendorf tube, centrifuged and the supernatant was removed. This was done separately for both organisms. There were a total of fifteen Eppendorf

tubes each (5 different time intervals and three treatment conditions). The treatments were Wash (saline, povidone iodine and sodium hypochlorite (W)), WPB (wash, polymyxin B and bacitracin) and WPBT (wash, polymyxin B, bacitracin and 250 µg EGCG-S) . For the wash condition 70 µl was prepared and combined with 30 µl of broth. WBP was made by mixing 70 µl of wash, 10 µl of 10X stock bacitracin, 10 µl of 10X stock polymyxin B and 10 µl of broth. WPBT solution was prepared by adding 70 µl of W, 10 µl of 10X bacitracin, 10 µl of 10X polymyxin B and 10 µl (10X 250 µg /ml). Once the treatments were added to the Eppendorf tube with the microorganism, a timer was set. At the end of each time interval, 100 µl of the microorganism was removed and spread aseptically on a TSA plate. The plates were then incubated at 37°C and checked the following day.

c. Colony Forming Units (CFU) Assay

Overnight cultures for *S. aureus* and *S. epidermidis* were made. The optical density (OD) was measured the next day by putting 1 ml of the overnight culture into a cuvette and taking a reading with a Spectronic 20 at 600 nm. The overnight cultures were diluted with broth until an OD of 0.2 was reached. 50 µl of cells (OD 0.2) were transferred to an Eppendorf tube and centrifuged at 5,000 rpm for 10 minutes. The supernatant was removed, and treatments were added to the pellet. A control group was constructed by adding 100 µl of broth to the pellet. The treatment groups WPB and WPBT were prepared by mixing 80 µl of W+ 10 µl of P + 10 µl of B (WPB), 70 µl of W + 10 µl of P + 10 µl of B + 10 µl of T (WPBT). Once treatments were placed on the cells, the Eppendorf tubes were vortexed. A timer was then set for 1 hour. When an hour of treatment was finished serial dilutions were carried out. The dilutions were 10^{-2}, 10^{-3} and

10^{-4}. The first dilution (10^{-2}) was done by removing 10 µl of cells from the Eppendorf tube that had the treated cells and putting it into 990 µl of DI H_2O. Ten µl was then taken from 10^{-2} and added to 990 µl of DI H_2O for 10^{-3} dilution from 10^{-3} dilution 10 µl was removed and added to another tube with 990 µl of H_2O for a 10^{-4} dilution. The tubes were vortexed after each dilution step. Volumes of 100 µl of 10^{-3} and 10^{-4} were plated and spread on TSA plates. The plates were taped and incubated at 37°C, results were recorded the next day. The colonies were counted, and percent inhibition was calculated by the following formula:

$$\%\ Inhibition = \frac{(CFU\ of\ Control - CFU\ of\ Treated)}{CFU\ of\ Control} \times 100$$

d. Colony Forming Units for Four-time Intervals

Overnight cultures were prepared for *S. aureus*, *S. epidermidis* and *P. aeruginosa*. The conditions for treatment were: control (100 µl saline + 200 µl DI H_2O + 100 µl EtOH + 600 µl TSB), WPB (100 µl wash + 100 µl 10X polymyxin B +100 µl 10X bacitracin + 100 µl EtOH + 600 µl TSB), WBPT (100 µl wash + 100 µl 10X of polymyxin B + 100 µl of 10X bacitracin + 100 µl of 10X 250 µg /ml EGCG-S + 600 µl TSB). Fifty µl of each microorganism was obtained and put into Eppendorf tubes three times. A combination of the bacteria was prepared by acquiring 16.6 µl of each microorganism in an Eppendorf tube with three replicates. After obtaining 50 µl of cells for all conditions, the Eppendorf tubes with the cells were centrifuged at 13.3 rpm for 10 minutes. The supernatant was removed and 50 µl of treatment was added for each condition and mixed vigorously. A timer was set for 1 minute, 5 minutes, 30 minutes and 1 hour. After each time interval, 10 µl of treated cells were removed and put into 990 µl of DI water. Further serial dilutions

were carried until 10^{-6} and 10^{-7}. These two dilutions were plated, spread and incubated at 37°C. A countable range of CFU's were obtained the following day for 10^{-6} dilution. Zero colonies were observed for *P. aeruginosa*; the experiment was repeated and a dilution of 10^{-5} was plated.

e. Resazurin Assay for Detection of Biofilm Inhibition

Overnight cultures were prepared for all three microorganisms and diluted to a 0.3 optical density. The combination of all three bacteria was prepared by combining 1 ml of each of the diluted stocks for the three organisms in a separate tube. A 96-well plate was used for this experiment. A positive control was set up for each microorganism (combination included) by adding 10 µl of DI H_2O to 90 µl of bacteria. Negative control consisted of 100 µl of TSB. WPB treatment was set up by taking 70 µl of diluted bacteria, centrifuging at 13.3 rpm for 10 minutes, removing the supernatant and adding 10 µl of Wash, Polymyxin B and Bacitracin. 70 µl of diluted cells for WPBT were also obtained and centrifuged for 10 minutes. The supernatant was removed and 10 µl of wash, polymyxin B, bacitracin and 10X 250 µg /ml EGCG-S were added to the pellet and vortexed; the mixed sample was then pipetted to a well. Three repeats were done for each condition. After all conditions were set up, parafilm was added to the 96 well plate. The plate was placed in the 37°C incubator and biofilm was allowed to grow for four days. The suspension was removed from the plate. After removing the liquid, the wells were washed with 100 µl of 1X PBS. A 1X stock of resazurin was prepared by diluting 1 ml of 2mM stock (5 mg resazurin powder + 10 ml of 1x PBS) with 9 ml of PBS. 120 µl of resazurin was added to each sample. The plate was then covered with aluminum foil and left in a 4°C for 24 hours to allow a full reaction to take place. The next day a

microplate reader was used to measure fluorescence with excitation and emission wavelength at 560 nm and 590 nm respectively. Relative light units (RLU) were measured and percent inhibition was calculated according to the following formula:

$$\% \, Inhibition = \frac{(RLU \; of \; Control - RLU \; of \; Treated)}{RLU \; of \; Control} \times 100$$

6. Virulent Factors

a. Protease Assay

Protease assay protocols were modified from a previous study completed by Tran et al (2014). Overnight cultures were prepared and diluted to 0.1, 0.3, and 0.6 at $OD_{600.}$ of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, and a combination of all three were incubated at 37°C for 8-10 hours. For the untreated condition, 500 µl of cells were used with two repeats per dilution. The treated condition 450 µl of cells were used with 50 µl of 10X 250 µg /ml EGCG-S with two repeats per dilution. The study was conducted over 4 days. Each day 250 µl of azocasein in buffer (consisting of 0.05 M TRIS HCl and 0.5 mM CaCl2) was added and incubated at room temperature for 15 minutes, followed by the addition of 375 µl of 10% trichloroacetic acid (TCA). The solution was then centrifuged at 10,000 rpm for 10 minutes. The absorbance of the supernatant was measured with a Tecan Infinite Pro Micro-Plate Reader at $OD_{400.}$ The calculation for percentage of inhibition was determined using the following equation: [(control untreated OD_{400} – treated OD_{400})/ control untreated OD_{400})] X 100.

b. Elastase Assay

Elastase assay protocols were modified from a previous study completed by Tran et al (2014). Overnight cultures were prepared and diluted to 0.1, 0.3. and 0.6 at $OD_{600.}$ For

the untreated condition, 500 µl of cells were used with two repeats per dilution. For the treated condition 450 µl of cells were used with 50 µl of 10X 250 µg /ml EGCG-S with two repeats per dilution. The study was conducted over 4 days. Each day 50 µl of supernatant from the untreated and treated cells were added into 450 µl of Elastin Congo Red Buffer (ECR) (consisting of 100 mM Tris and 1 mM $CaCl_2$ at pH 7.5) containing 10 mg of ECR and incubated with shaking at 37°C for 3 hours. After three hours, the reaction was stopped by adding 500 µl of 0.7 M sodium phosphate buffer (pH 6.0). Tubes were then placed in an ice water bath at 4°C for 15 minutes and centrifuged to remove the insoluble ECR. The absorbance of the supernatant was measured with a Tecan Infinite Pro Micro-Plate Reader at OD_{495}. Calculation for percentage of inhibition was determined using the following equation: [(control untreated OD_{495} – treated OD_{495})/ control OD_{495})] X 100.

c. Evaluation of the Current Treatment with and without EGCG-S

The same procedure for protease and elastase assay as mentioned above was followed for this study. Overnight cultures were prepared and diluted to an OD of 0.6. Two replications were made for each condition, for 4 days. The four conditions were control, wash (W), wash with polymyxin B (P) and bacitracin (B) (WPB), and wash with PB and 250 µg /mL EGCG-S (WPBT). Each condition had 350 µl of the respective cells. The control condition consisted of 150 µl of H_2O. The wash condition had 56.5 µl of saline, 78.5 µl of sodium hypochlorite, and 15 µl of povidone. The wash with PB solution consisted of 50 µl of saline, 78.5 µl of Dakin's, 15 µl of povidone, 5µl of 100X polymyxin, and 5µL of 100X of bacitracin. The wash with PB and Tea solution consisted

of 55 µl of saline, 78.5 µl of sodium hypochlorite, 15 µl of povidone, 5µl of 100X polymyxin and bacitracin, and 50µl of 10X 250 µg /mL EGCG-S.

Results and Discussion

This study consisted of the use of three microorganisms: Gram positive bacteria: *Staphylococcus aureus* and *Staphylococcus epidermidis*; and Gram-negative bacteria *Pseudomonas aeruginosa.* These three bacteria are known to be the most prominent during the pathogenesis of prosthetic joint infections (PJI). Several experiments were carried out to establish the potential usage of EGCG-S with current methods to enhance the treatment of PJIs.

1. Gram Stain and Morphological Arrangement of Three Bacteria

Gram stain is a technique used to divide bacteria into two groups. Clinically, it can be used as a rapid diagnostic test. Gram-positive bacteria contain a thick peptidoglycan layer along their cell wall. During the staining process they retain the primary dye; crystal violet. Figures 1a and 1b show *Staphylococcus aureus* and *Staphylococcus epidermidis* as being gram positive (purple) cocci, with grape-like clusters. *Pseudomonas aeruginosa* shown in Figure 1 represents the characteristics of a gram-negative species (pink/red). The observed morphology of *P.aeruginosa* are single pink/red bacilli. Gram negative bacteria have a thinner peptidoglycan layer than gram-positive bacteria, due to this characteristic they are unable to retain the primary dye during the decolorization process; therefore, acquiring the secondary stain safranin. Gram-negative species are noted as being more pathogenic than gram-positive species, they contain an additional outer membrane. This extra layer of protection acts as a virulent factor; releasing endotoxins (Thairu, Nasir, & Usman, 2014). It is important to know the gram status of bacteria as they may respond differently to treatments.

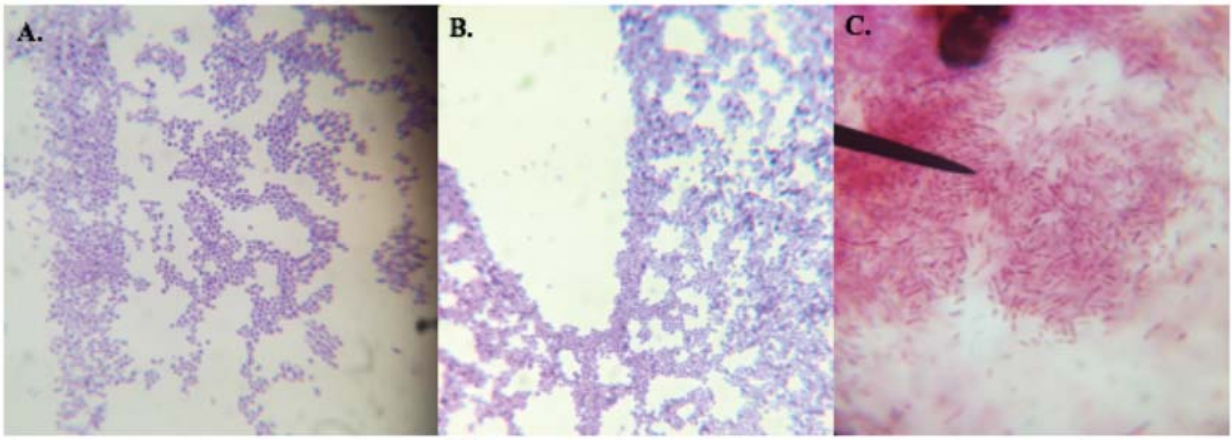


Figure 1. Gram-Stain a 1,000x TM. **(a)** *S. aureus* displaying gram-positive characteristics. Grape-like clusters of cocci are also present. **(b).** *S. epidermidis* showing gram positive characteristics. Grape-like clusters of cocci are also present **(c)** *P. aeruginosa* displaying gram-negative characteristics. Single bacilli can also be seen.

2. <u>Disk Diffusion Test</u>

Disk diffusion test to measure the zone of inhibition (ZOI) was used to evaluate the effectiveness of different compounds used for treating PJI. The purpose of the first disk diffusion test was to measure the effectiveness of the surgical wash components (wash, saline, povidone and sodium hypochlorite) on inhibiting cell growth (Figures 2-5). For *S. aureus*, *S. epidermidis*, *P. aeruginosa* and a combination of all three; saline and wash (saline, povidone and sodium hypochlorite combined) had no effect on any of the microorganisms. Povidone worked the best for *S. aureus* and *S. epidermidis* with a ZOI of 20.5 mm and 21mm respectively; followed by sodium hypochlorite, ZOI 12 mm (*S. aureus*) and 12.5 mm (*S.epidermidis)*. Sodium hypochlorite worked slightly better (11mm) than povidone (10 mm) on *P. aeruginosa.* For the combination of all three bacteria povidone and sodium hypochlorite exhibited similar results for ZOI with *P.aeruginosa,* having ZOI 21 mm for povidone and 12.5 mm sodium hypochlorite. Povidone and sodium hypochlorite have the best inhibitory effect. These data suggest that perhaps some of the components that make up the wash should be increased to a higher

concentration or exchanged (particularly saline) with another compound that has greater antibacterial capability.

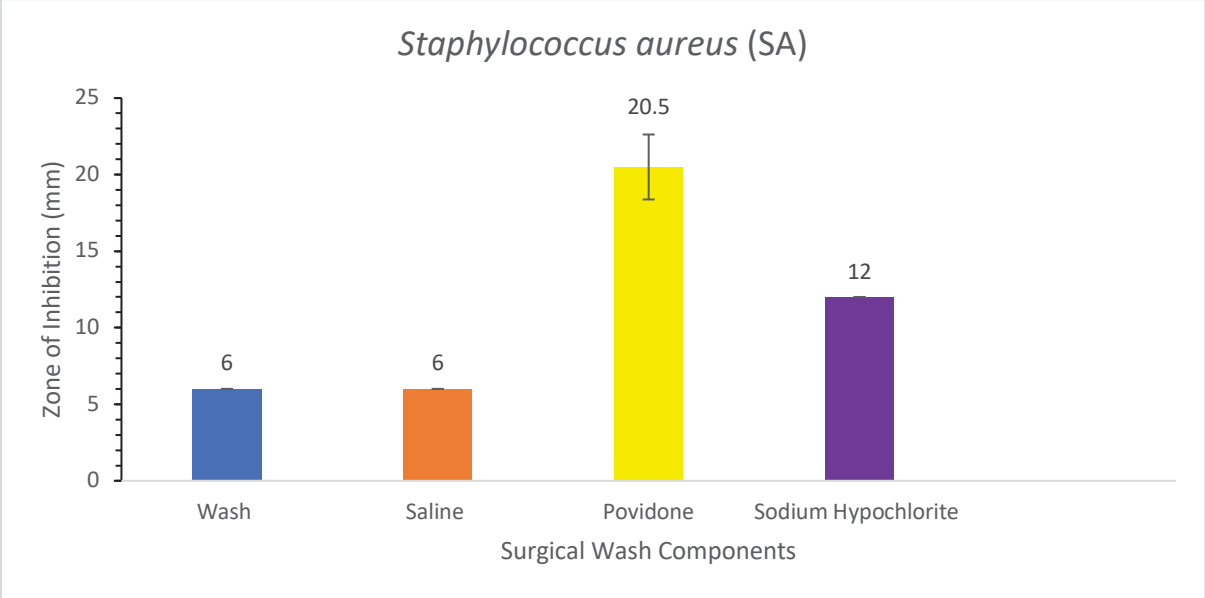


Figure 2. Zone of Inhibition Method for *S. aureus* with the use of Surgical Wash Components. The zone of inhibition was measured for a disk containing all the surgical wash components (Wash) and a disk containing those components separately. A 6 mm measurement was the length of the disk itself, indication that no inhibition occurred.

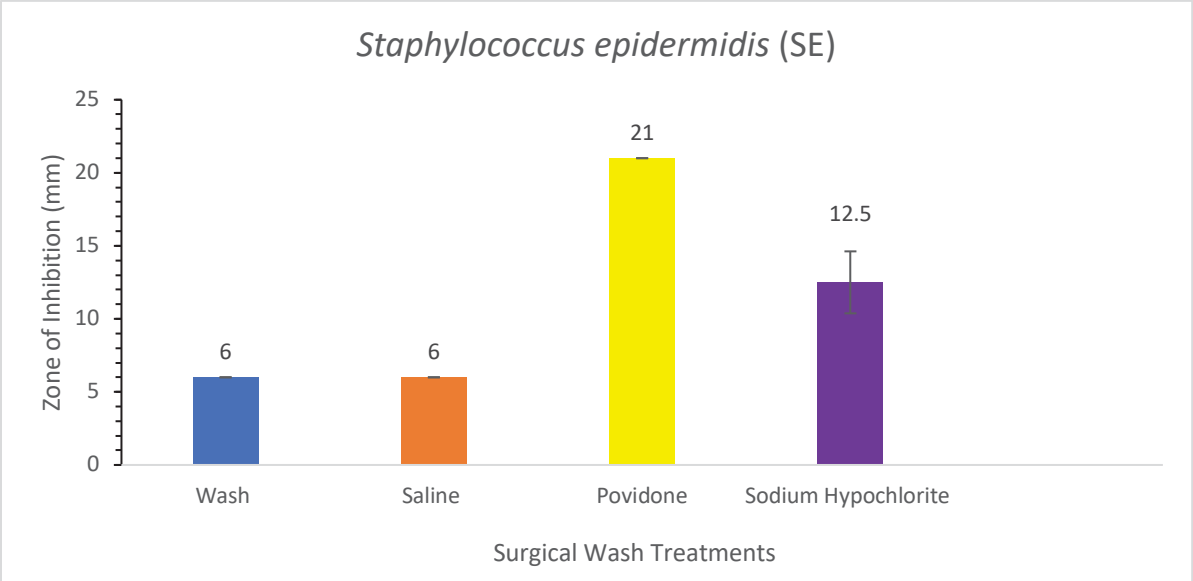


Figure 3. Zone of Inhibition Method for *S. epidermidis* with the use of Surgical Wash Components. The zone of inhibition was measured for a disk containing all the surgical wash components (Wash) and a disk containing those components separately. A 6 mm measurement was the length of the disk itself, indication that no inhibition occurred.

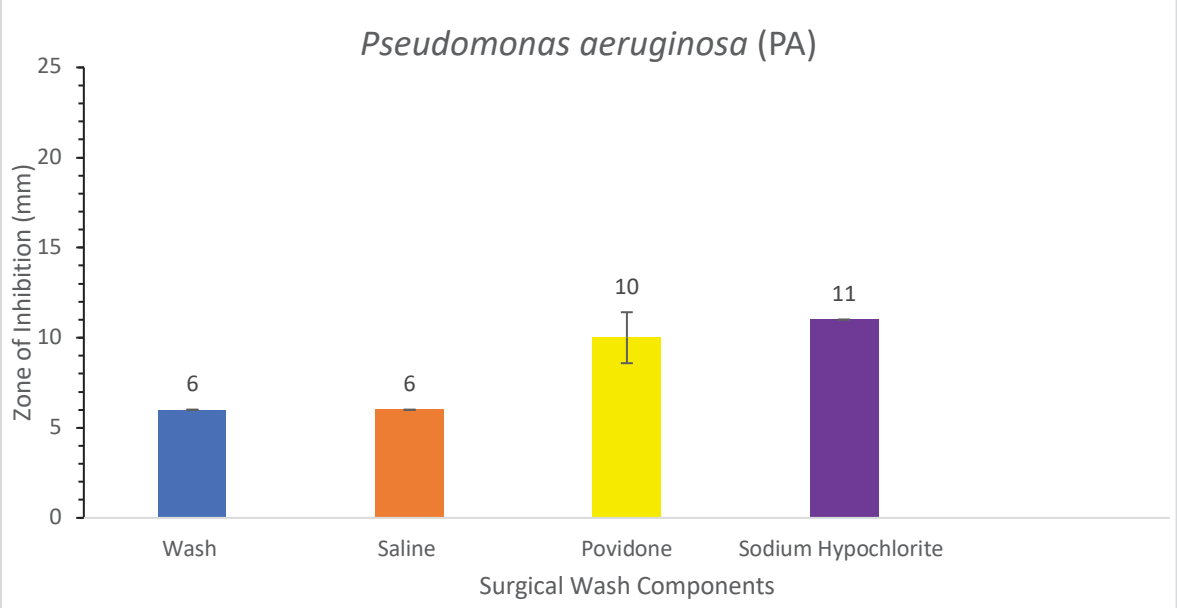


Figure 4. Zone of Inhibition Method for *P. aeruginosa* with the use of Surgical Wash Components. The zone of inhibition was measured for a disk containing all the surgical wash components (Wash) and a disk containing those components separately. A 6 mm measurement was the length of the disk itself, indication that no inhibition occurred.

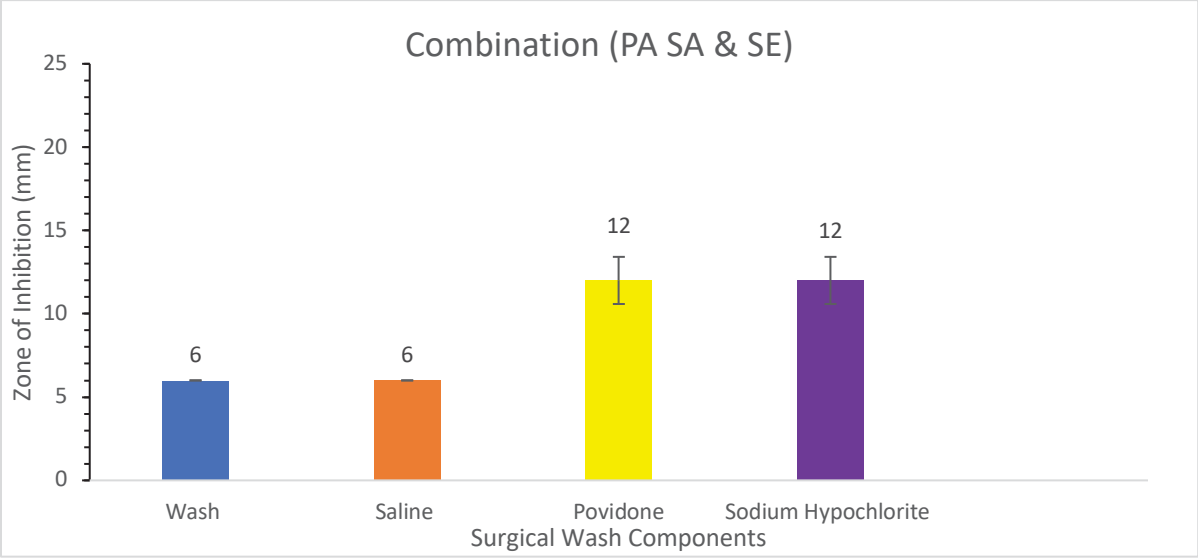


Figure 5. Zone of Inhibition Method for all three bacteria with the application of Surgical Wash Components. The zone of inhibition was measured for a disk containing all the surgical wash components (Wash) and a disk containing those components separately. A 6 mm measurement was the length of the disk itself, indication that no inhibition occurred.

The second disk diffusion study surveyed the current antibiotics used in treating joint infections and the tea polyphenol EGCG-S (Figures 6-9). Disk diffusion results using ZOI for the evaluation of the antibacterial effectiveness were divided into three categories: susceptible, intermediate and resistant as see in table 1. In this study the current surgical concentration for Polymyxin B (19.8 mg/10 ml) and Bacitracin (22.5 mg/10 ml) were measured and represented as 100X. A concentration of 10X and 1X were also made. For *S. aureus* (Figure 6) 100X bacitracin worked well in inhibiting the bacteria, the ZOI was large enough for the microorganism to be considered susceptible. *S. aureus* was resistant to three concentrations of polymyxin B (100x, 10x and 1x). An intermediate measurement for ZOI was shown for commercial polymyxin B (300 μg) on *S. aureus*. When EGCG-S was added to the 100X concentration of bacitracin and polymyxin B (BPT) the bacteria were susceptible. This study suggested that EGCG-S may have enhanced the activity of polymyxin B. To further test this, a combination of polymyxin B with EGCG-S and polymyxin B with bacitracin would have to be examined. The current concentration of bacitracin had an intermediate effect on targeting *S. epidermidis* (Figure 7). A small zone of inhibition was seen when a concentration of 100X polymyxin was used. This small zone of inhibition was not significant and still fell under the resistant category. Commercial polymyxin B displayed results for antimicrobial susceptibility. Previous studies, showed that commercial bacitracin (10 μg) on *S. epidermidis* had sensitive results (18 mm zone of inhibition). The data gathered here can lead us to infer that the concentrations used in treatment when targeting a population of *S. epidermidis* are not effective. Antibiotic treatments used to treat *P. aeruginosa* are also not effective. The only outcome for antimicrobial susceptibility was seen for the

commercial polymyxin B; an intermediate inhibition rate was observed for polymyxin 100X. The combination of all three bacteria (Figure 9) showed similar results to *P. aeruginosa*. The difference between the two was that when the antibiotics were combined with EGCG-S, we saw the largest zone of inhibition. Further testing for the combination group would involve examining EGCG-S's effect on polymyxin and bacitracin separately. Commercial polymyxin B also worked better at inhibiting the growth of bacteria than the prepared 100X, 10X and 1X concentrations. The concentration of the commercial polymyxin B was much lower hence, the opposite effect should have been seen. Examination of the preparation of commercial polymyxin would also have to be studied to understand why a larger ZOI is produced in comparison to samples that were prepared at a higher concentration.

Table 1. Measurements and color code for antibiotics.

Bacitracin	**Polymyxin B**
Susceptible >13 mm	Susceptible >12 mm
Intermediate 9-11 mm	Intermediate 9-11mm
Resistant < 8 mm	Resistant < 8mm

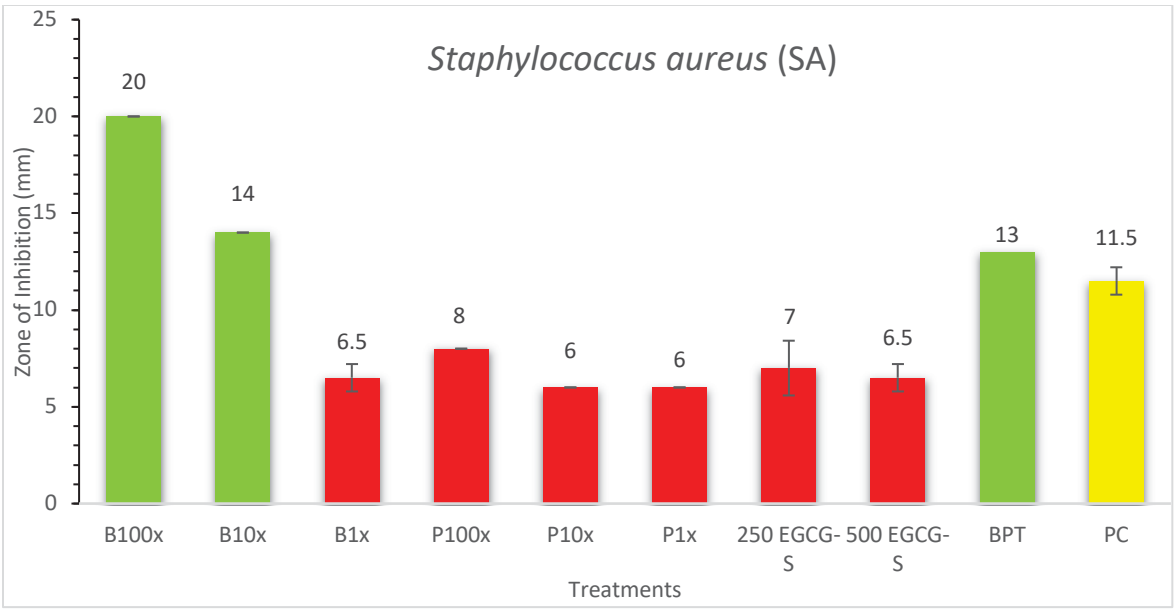


Figure 6. Zone of inhibition for current antibiotic treatment in addition to tea polyphenols for *S. aureus*. The current surgical concentration for Polymyxin B (19.8 g/10 ml) and Bacitracin (22.5 g/10 ml) were measured and represented as 100X. 10X and 1x were also observed. The effects of EGCG-S at a concentration of 250 µg /ml and 500 µg / ml can also be seen. The current concentration of polymyxin B and bacitracin were combined with 250 µg / ml EGCG-S, (BPT). A commercial disk of polymyxin (300 µg) (PC) was also tested.

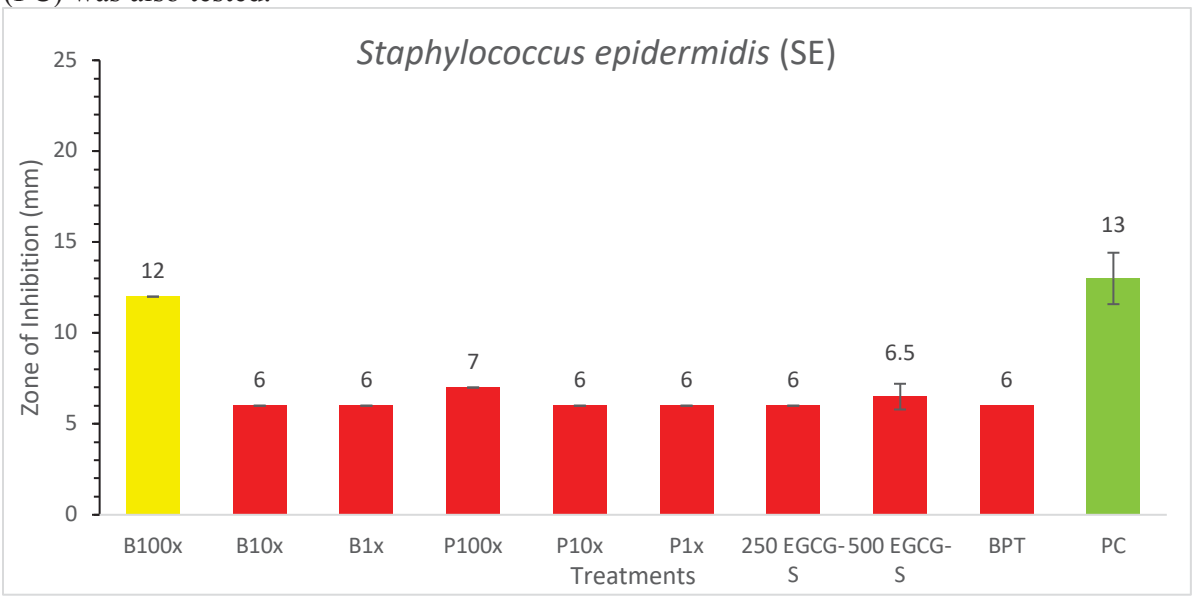


Figure 7. Zone of inhibition for current antibiotic treatment in addition to tea polyphenols for *S. epidermidis*. The current surgical concentrations for Polymyxin B (19.8 g/10 ml) and Bacitracin (22.5 g/10 ml) were measured and represented as 100x. 10x and 1x were also observed. The effects of EGCG-S at a concentration of 250 µg /ml and 500 µg /ml can also be seen. The current concentration of polymyxin B and bacitracin were combined with 250 µg / ml EGCG-S, (BPT). A commercial disk of polymyxin (300 µg) (PC) was also tested.

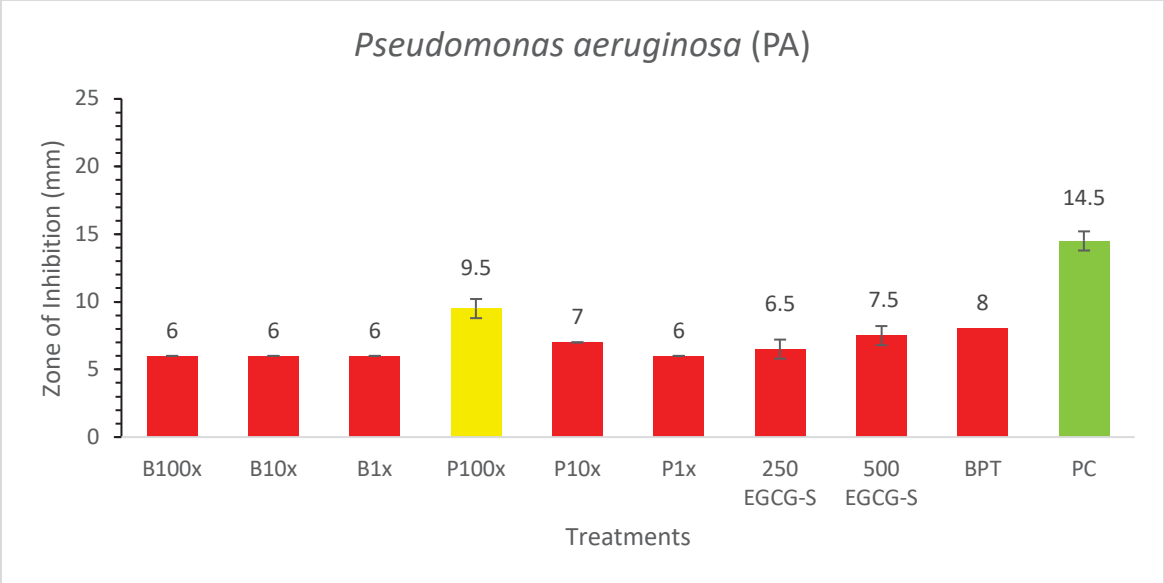


Figure 8. Zone of inhibition for current antibiotic treatment in addition to tea polyphenols for *P. aeruginosa*. The current surgical concentration for Polymyxin B (19.8 g/10 ml) and Bacitracin (22.5 g/10 ml) were measured and represented as 100x. 10x and 1x were also observed. The effects of EGCG-S at a concentration of 250 µg /ml and 500 µg / ml can also be seen. The current concentration of polymyxin B and bacitracin were combined with 250 µg /ml EGCG-S, (BPT). A commercial disk of polymyxin (300 µg) (PC) was also tested.

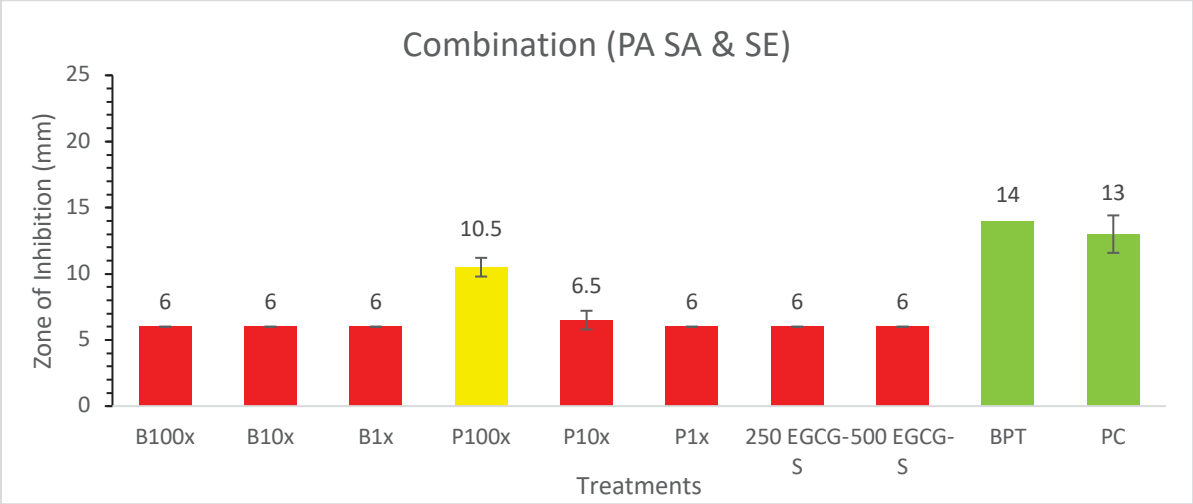
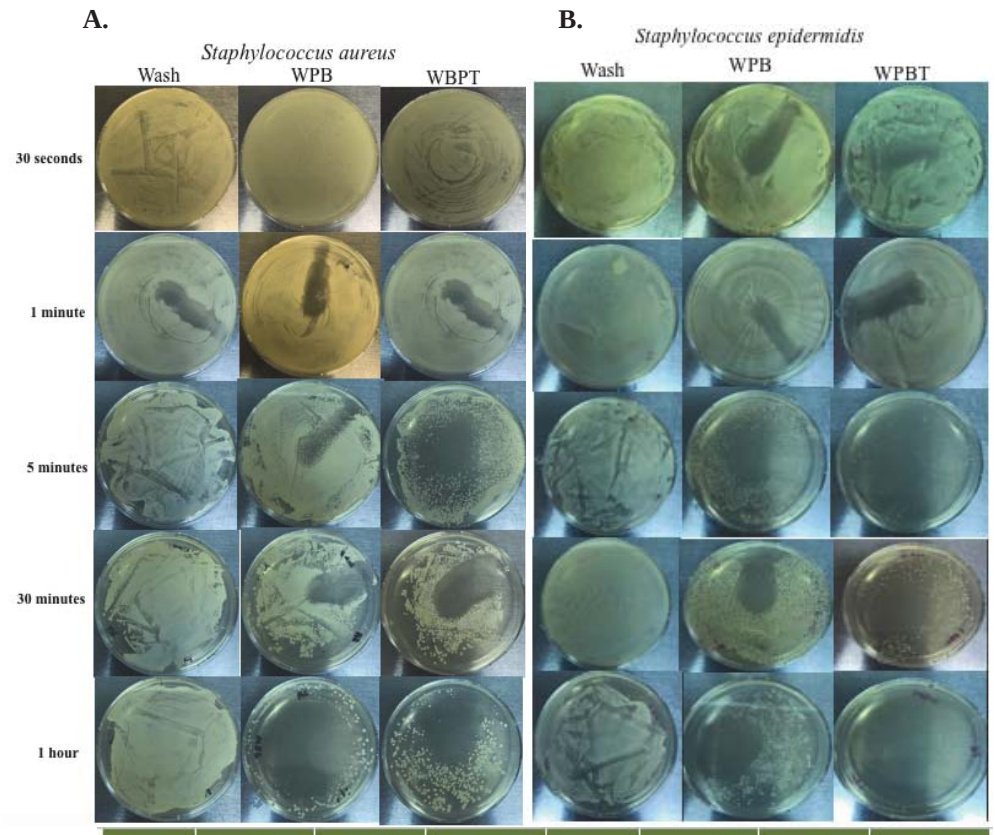


Figure 9. Zone of inhibition for current antibiotic treatment in addition to tea polyphenols for three bacteria. The current surgical concentration for Polymyxin B (19.8 g/10 ml) and Bacitracin (22.5 g/10 ml) were measured and represented as 100x. 10x and 1x were also observed. The effects of EGCG-S at a concentration of 250 µg /ml and 500 µg / ml can also be seen. The current concentration of polymyxin B and bacitracin were combined with 250 µg / ml EGCG-S, (BPT). A commercial disk of polymyxin (300 µg) (PC) was also tested.

3. Timed Course Study to Evaluate the Effectiveness of Different Treatments used for PJI

Overnight cultures for *S. aureus* and *S. epidermidis* were prepared to determine the time that would be most effective when administering treatment and to also assess whether EGCG-S had any observable differences when combined with the current treatment. Figures 10a and 10b show that at 30 seconds and 1 minute for both organisms in all the treatment groups, the plates are almost confluent indicating that these times are not effective when treating PJIs. At 5 minutes, wash did not make any difference for both organisms; a large difference can be seen between both organisms, especially with WPB (wash, polymyxin B & bacitracin) and WPBT (wash, polymyxin B, bacitracin and 250 µg /ml EGCG-S) used on *S. epidermidis*. This time frame also allows us to be able to count the colonies and compare, WPBT has a remarkable difference when compared with the current treatment. At 30 minutes and 1 hour a similar trend can be seen with the least number of colonies being observed for WPBT. The number of colonies also continues to decrease as time increases for WPB and WPBT. For one-hour treatment with WPBT, there is only one colony observed on the plate for *S.epidermidis* From these results we can infer that the current time restraint, usually a few seconds is not efficient at targeting bacterial species. Five minutes is the minimum time for antibiotics and EGCG-S to be most advantageous. Addition of EGCG-S indicated its synergistic inhibition effect on the current treatment.



C.

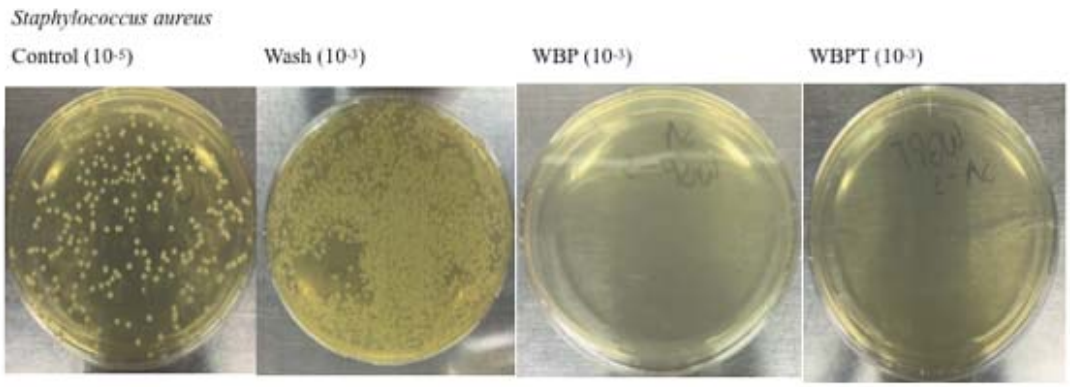
Time	Wash(CFU)	WBP(CFU)	WBPT(CFU)	Time	Wash(CFU)	WBP(CFU)	WBPT(CFU)
5 minutes	-	1,960	1,230	5 minutes	-	1,518	71
30 minutes	-	364	144	30 minutes	-	2,567	263
1 hour	-	167	640	1 hour	-	898	1

Figure 10. Determination of Optimal Time Period for Treatment. (a) *S. aureus* treated with Wash, WPB and WPBT for 30 seconds, 1 minute, 5 minutes, 30 minutes and 1 hour. The bacteria were then grown on tryptic soy agar plates. **(b).** *S. epidermidis* treated with Wash, WPB and WPBT for 30 seconds, 1 minute, 5 minutes, 30 minutes and 1 hour. The bacteria were then grown on tryptic soy agar plates. **(c).** The table exhibits the times and treatment conditions that colonies were able to be counted.

4. Colony Forming Units (CFU) Assay

This experiment was done as a quantitative method to allow us to measure cell viability and further measure the enhancing capabilities of EGCG-S on current PJI treatment. The cells were treated with wash, WPB and WPBT for one hour. The control group had no treatment, cells were resuspended in broth. After treatment the cells were plated on tryptic soy agar (TSA) plates. Figure 11 shows that for *S. aureus,* the wash had a 98% inhibition followed by 100% with the addition of polymyxin and bacitracin. When EGCG-S was added to the wash in combination with polymyxin and bacitracin a 100% inhibition was also observed. Similar results were observed for *S. epidermidis* and *P. aeruginosa*, (Figure 12 and Figure 13, respectively). The wash results varied between these two organisms (*S.epidermidis* and *P.aeruginosa*); a 93% inhibition for *S. epidermidis* and 62% inhibition for *P. aeruginosa* were also observed. For one-hour treatment, the effect of WBP and WBPT did not show any difference for the CFU assay (0 CFU). Further experiments would involve conducting the experiment with lower dilutions at the one-hour time frame. This could possibly allow us to obtain a countable range and determine whether there is a major difference when EGCG-S is added to the two antibiotics.

A.



B.

Treatment	Colony Forming Units/mL
Control	1.92×10^7
Wash	3.76×10^4
WBP	0
WBPT	0

C.

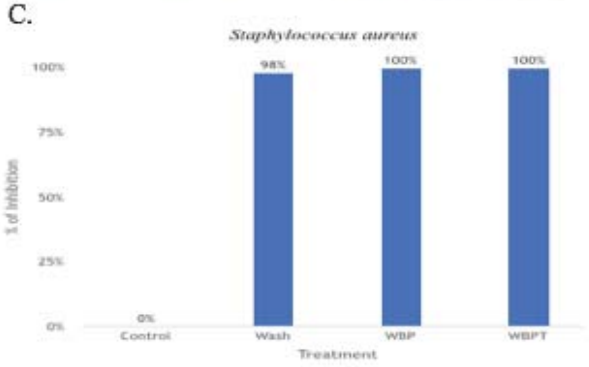
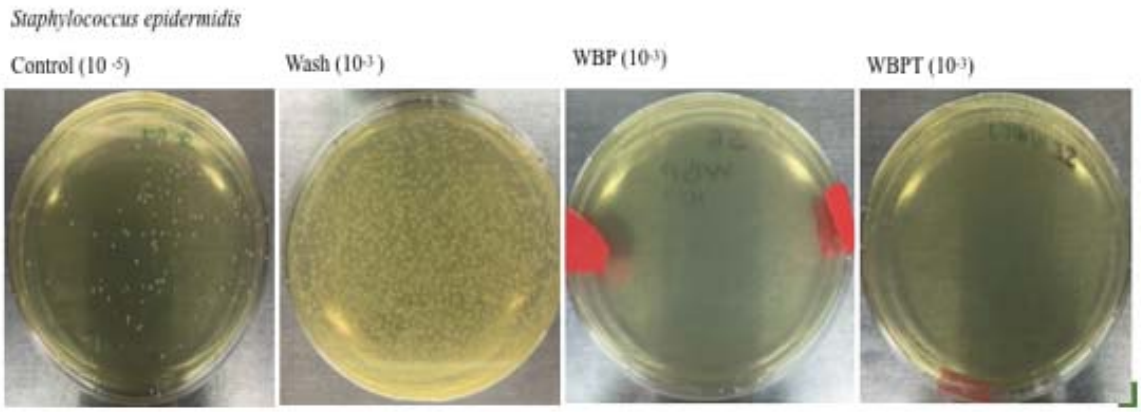


Figure 11. The Effect of Surgical Wash Components with the Addition of EGCG-S on CFU for *S. aureus*. (a). Tryptic soy agar plates that the bacteria were grown on, colony forming units can be seen on the control and wash plate. WBP (wash, polymyxin and bacitracin) and WBPT (wash, bacitracin and polymyxin) plates appear to be transparent with no colony forming units. **(b).** Table shows the total plate count for each condition. **(c).** Percent inhibition for the treated groups.

A.



B.

Treatment	Colony Forming Units/mL
Control	6.3 x 10^6
Wash	4.6 x 10^5
WBP	0
WBPT	0

C.

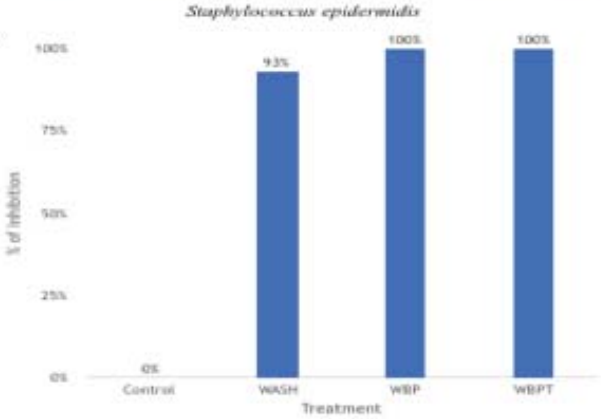
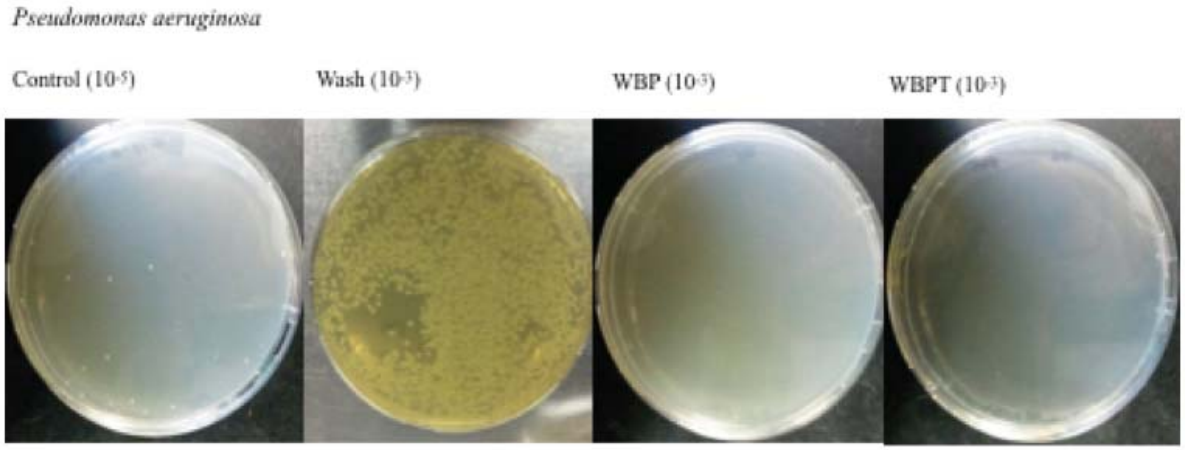


Figure 12. The Effect of Surgical Wash Components with the Addition of EGCG-S on CFU for *S. epidermidis*. (a). Tryptic soy agar plates that the bacteria were grown on, colony forming units can be seen on the control and wash plate. WBP (wash, polymyxin and bacitracin) and WBPT (wash, bacitracin and polymyxin) plates appear to be transparent with no colony forming units. **(b).** Table shows the total plate count for each condition. **(c).** Percent inhibition for the treated groups.

A.



B.

Treatment	Colony Forming Units/mL
Control	1.3×10^6
Wash	4.96×10^5
WBP	0
WBPT	0

C.

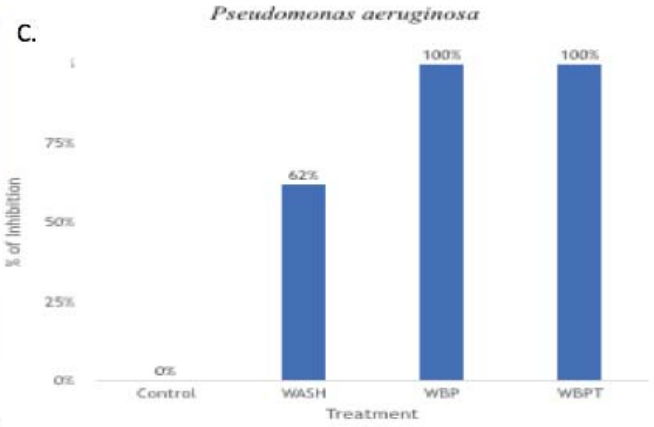


Figure 13. The Effect of Surgical Wash Components with the Addition of EGCG-S on CFU for *P. aeruginosa* (a). Tryptic soy agar plates that the bacteria were grown on, colony forming units can be seen on the control and wash plate. WBP (wash, polymyxin and bacitracin) and WBPT (wash, bacitracin and polymyxin) plates appear to be transparent with no colony forming units. **(b).** Table shows the total plate count for each condition. **(c).** Percent inhibition for the treated groups.

An additional CFU assay was done for the time course study. It involved expanding on the results obtained in Figure 10. The two treatments that were shown to have the most effect on cell viability, WPB and WPBT, were used. In addition to *S. aureus* and *S. epidermidis*; *P. aeruginosa* and a combination of all three bacteria were also examined. The results show that for *S. aureus* EGCG-S had the most synergistic effect at the 1-hour mark with bacitracin and polymyxin (Figure 14). At 30 minutes EGCG-S with WBP also had a higher percent inhibition than WBP alone at that same time frame. A large standard deviation was present for WPB which suggested that data acquired is not reliable. At 5 minutes the tea polyphenol is seen to have no effect and at the 1-minute time frame WPB works slightly better. EGCG-S in combination with WPB is shown to have higher inhibitory effect for *S. epidermidis* at 5 minutes, 30 minutes and 1 hour. *P. aeruginosa* is impacted the most by EGCG-S at 1-hour treatment time (Figures 15 and 16). At 1 minute and 5 minutes WPB seems to have the most progress. When all the bacteria were grown together, EGCG-S had the best synergistic effect at 30 minutes and 1 hour (Figure 17). WPB worked better than WPBT at 1minute and 5 minutes. From the results obtained in Figure 10, it was expected that as time increased, the more cell death there would be and treatment with the addition of EGCG-S would have prime results. This trend is strictly not observed for *S.aureus, P.aeruginosa* and combination. Possible reasons for this could be that the population of cells was not consistent for the organisms resulting in fluctuating data. Dilution errors could have also played a role. A higher serial dilution was also used for this study, when compared to the results obtained for the effect of surgical wash components with the addition of EGCG-S on CFU (Figure 10) the results contradict; a dilution of 10^{-3} performed and the outcome was 0 colonies.

For timed treatment (Figures 14-17), a dilution of 10^{-3} was too low; 10^{-6} gave us the countable range. Future studies will involve starting with the same optical density for all cell populations and repeating the four-time frames to determine if treatment with the addition of EGCG-S is able to enhance the activity of the current treatment.

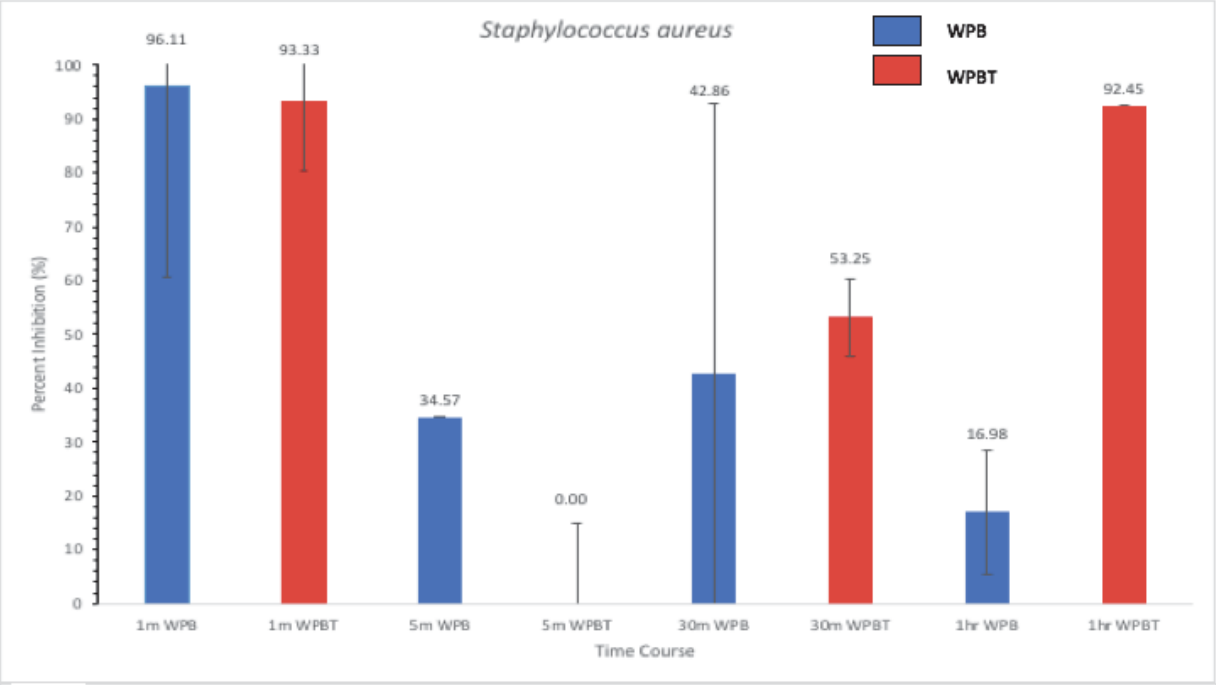


Figure 14. *S. aureus* CFU for Timed Treatments. *S. aureus* treated with WPB and WPBT for 1 minute, 5 minutes, 30 minutes and 1 hour. Treated cells were diluted to 10^{-6}.

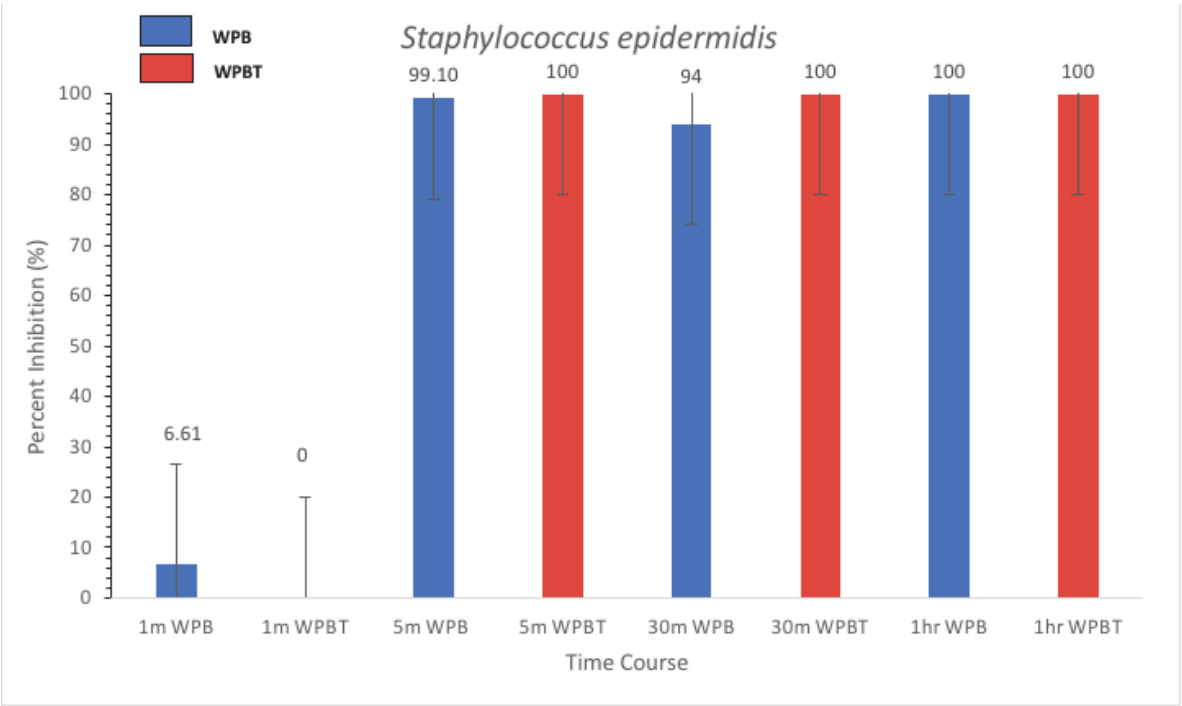


Figure 15. *S. epidermidis* CFU for Timed Treatments. *S. epidermidis* treated with WPB and WPBT for 1 minute, 5 minutes, 30 minutes and 1 hour. Once treated cells were diluted to 10^{-6}.

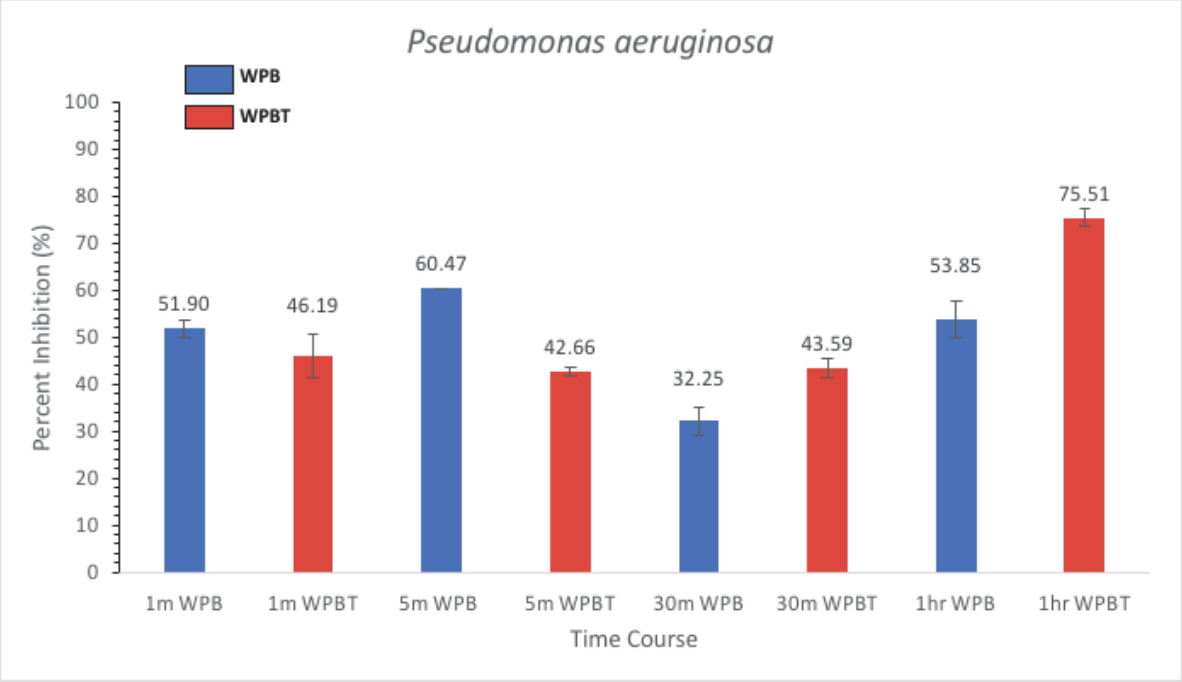


Figure 16. *P. aeruginosa* CFU for Timed Treatments. *P. aeruginosa* treated with WPB and WPBT for 1 minute, 5 minutes, 30 minutes and 1 hour. Treated cells were diluted to 10^{-5}.

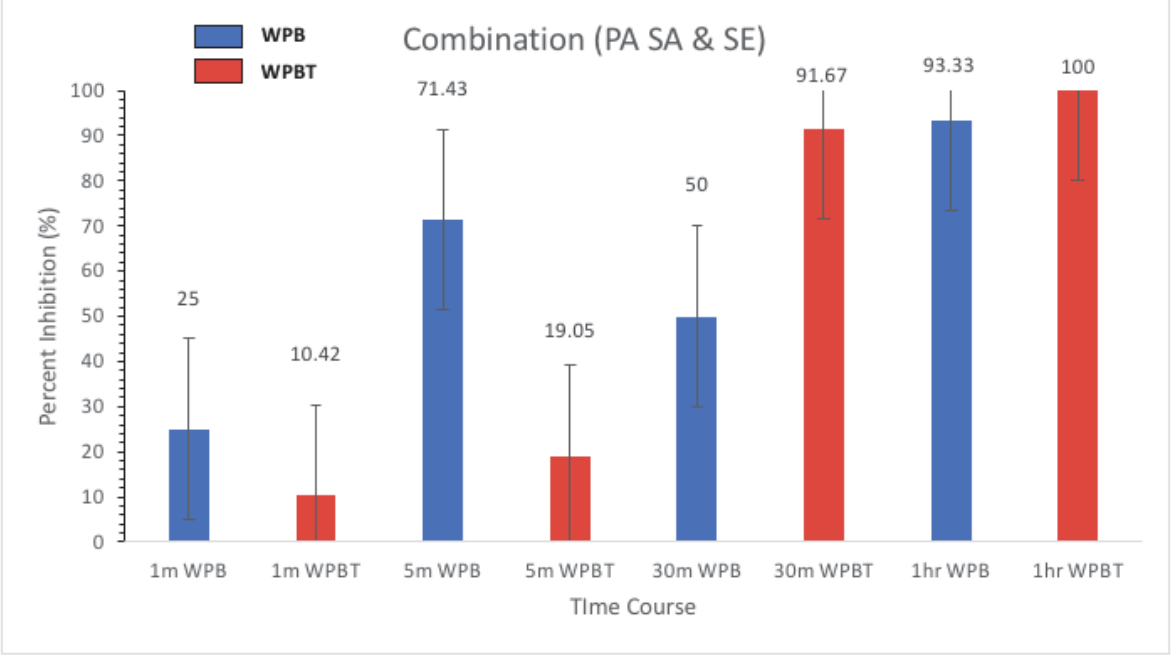


Figure 17. *S. aureus, S. epidermidis* and *P. aeruginosa* CFUs Timed Treatments. 1/3 of each bacterium were combined and treated WPB and WPBT. The treatment times were 1 minute, 5 minutes, 30 minutes and 1 hour. Treated cells were diluted to 10^{-5}.

5. Resazurin Assay for the Inhibition of Biofilm

Resazurin is a non-toxic stable stain used to measure cell viability. Cells that are metabolically active reduce non-fluorescent resazurin to the fluorescent product resorufin (Van den Driessche, Rigole, Brackman & Coenye, 2013). Results shown in Figure 18 indicate that WPBT is just as effective as WPB at treating biofilms caused by *S. aureus* and *S. epidermidis.* EGCG-S is shown as having a more considerable synergistic effect on *P. aeruginosa* biofilm than with WPB alone. For the combination treatment WPB has a slightly greater outcome than WPBT. Previous studies have shown the biofilm caused by *P. aeruginosa* to be the most difficult to treat (Chen, 2016). From our findings *P. aeruginosa* has been noted to rapidly accumulate and dominate a population when other microorganisms are present. This study suggests that EGCG-S can play a crucial role in targeting biofilms caused by *P. aeruginosa*.

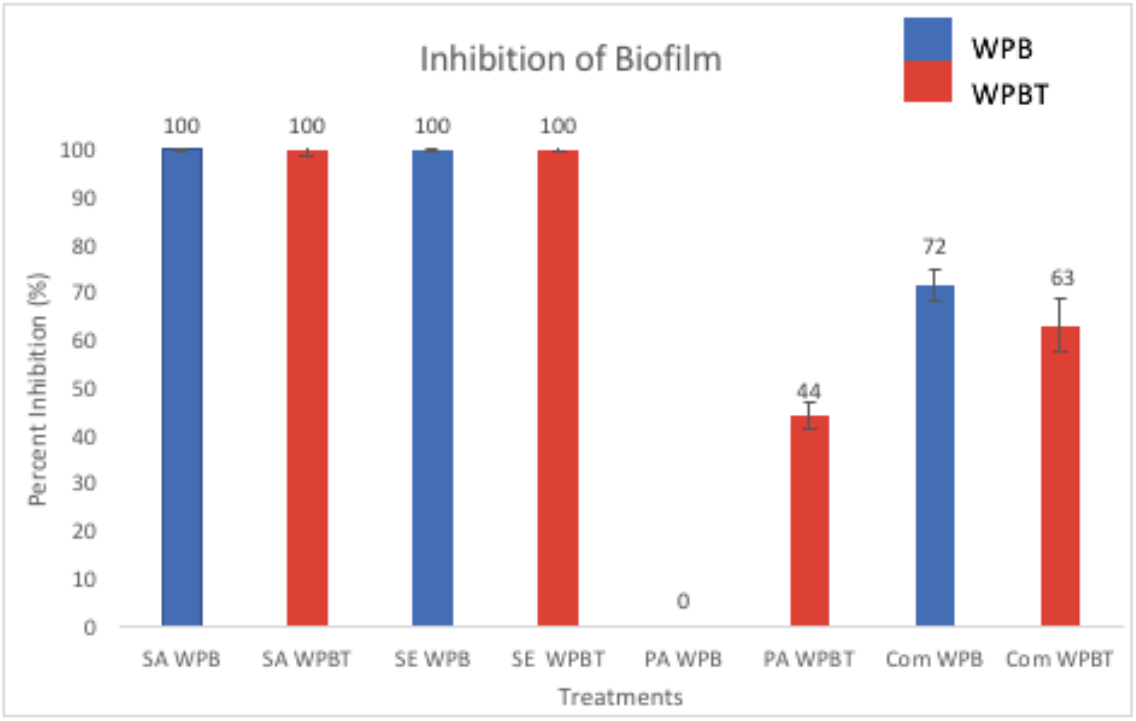


Figure 18. Resazurin Assay. The three predominant microorganisms in PJI were examined separately and in combination for biofilm inhibition. The two treatments used

to target the biofilm were WPB (wash, polymyxin B and bacitracin) and WPBT (wash, polymyxin, bacitracin and 250 μg EGCG-S).

6. Virulent Factors

Quorum sensing is a technique that gram-negative and positive bacteria use for group behavior. Autoinducers are released for communication allowing the population to persist and grow (Raffa et al., 2004). Protease and elastase are proteolytic enzymes that allow bacteria to invade colonize a host's tissue (Tran et., al). A compound that targets these enzymes could eliminate the destructiveness caused to the host.

Protease production was measured for the prominent microorganisms involved in causing prosthetic joint infection. Three optical densities were used to determine whether the production of protease is cell population dependent. Protease readings were taken during the course of four days to determine if biofilm density has any effect in the amount of protease production for each of the groups (S. *aureus*, *S. epidermidis, P. aeruginosa* and combination). The results are shown in Figures 19-22. Optical density of cells did not have a significant effect on the amount of protease production. For *S. aureus*, *S. epidermidis* and *P. aeruginosa* the amount of protease output increased on the first day and stayed consistent for days 2-4. When all three bacteria were combined, the greatest amount of protease production was during day 2. The amount of protease measured for the bacteria in combination was much more substantial than when the bacteria were existing amongst their own population. The results indicate that when the bacteria are in combination, the enzyme is enhanced. This mimics what would happen in a joint infection that has more than one species. The effect of EGCG-S was measured for bacteria that had been treated for two and four days (Figure 23). The most notable %

inhibition was observed for the bacteria in combination (Table 2). The % of inhibition was 78.03 % and 76.79 % for day 2 and day 4, respectively.

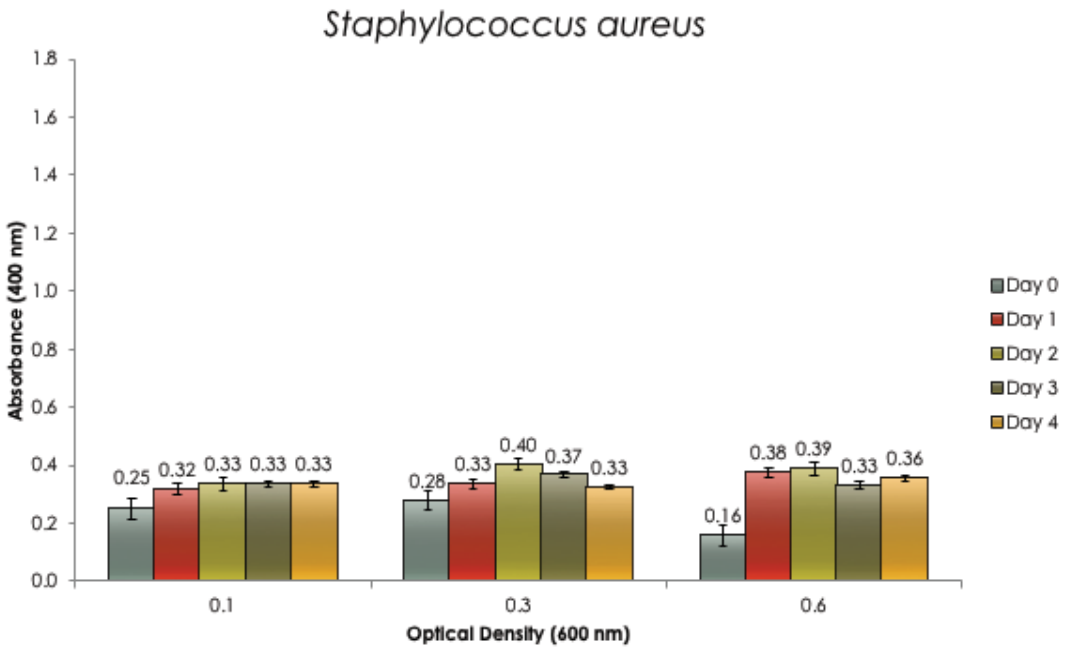


Figure 19. Protease assay for *S. aureus* during the course of four days. The production of protease was measured under three optical densities (0.1, 0.3 and 0.6).

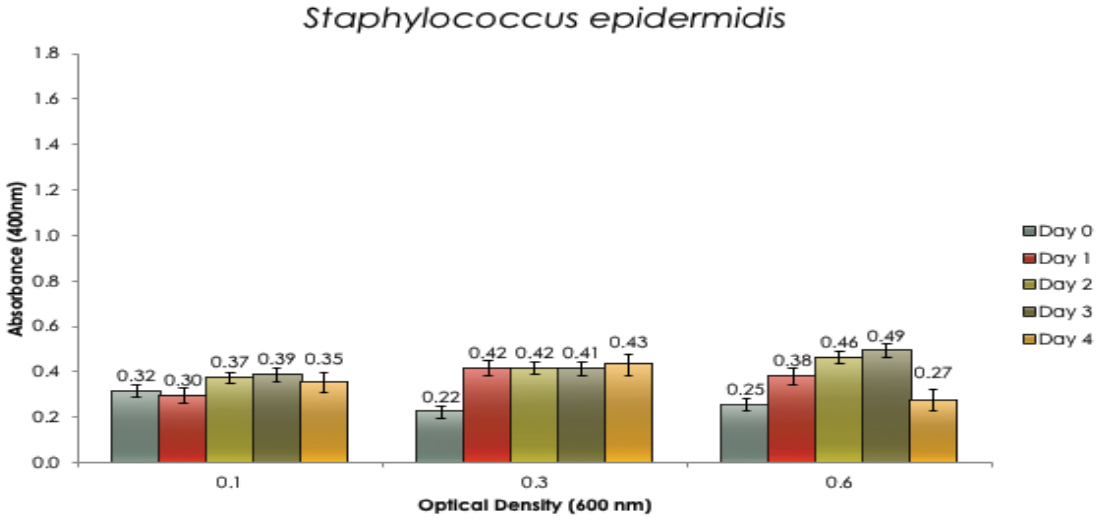


Figure 20. Protease assay for *S. epidermidis* during the course of four days. The production of protease was measured under three optical densities (0.1, 0.3 and 0.6) for the microorganism.

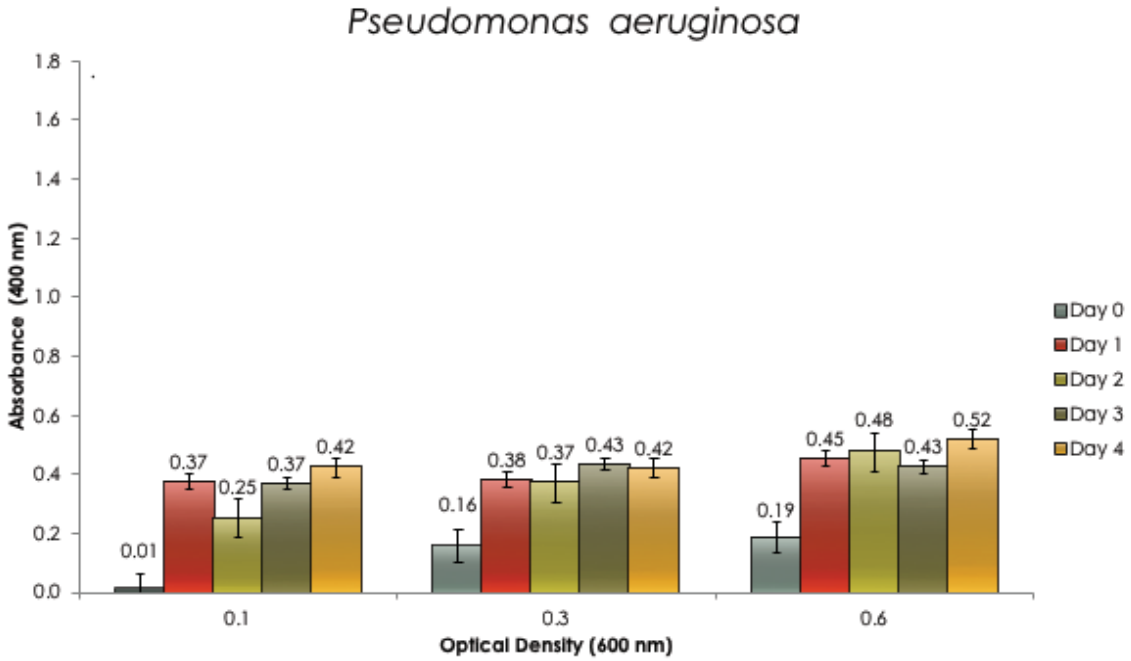


Figure 21. Protease assay for *P. aeruginosa* during the course of four days. The production of protease was measured under three optical densities (0.1, 0.3 and 0.6) for the microorganism.

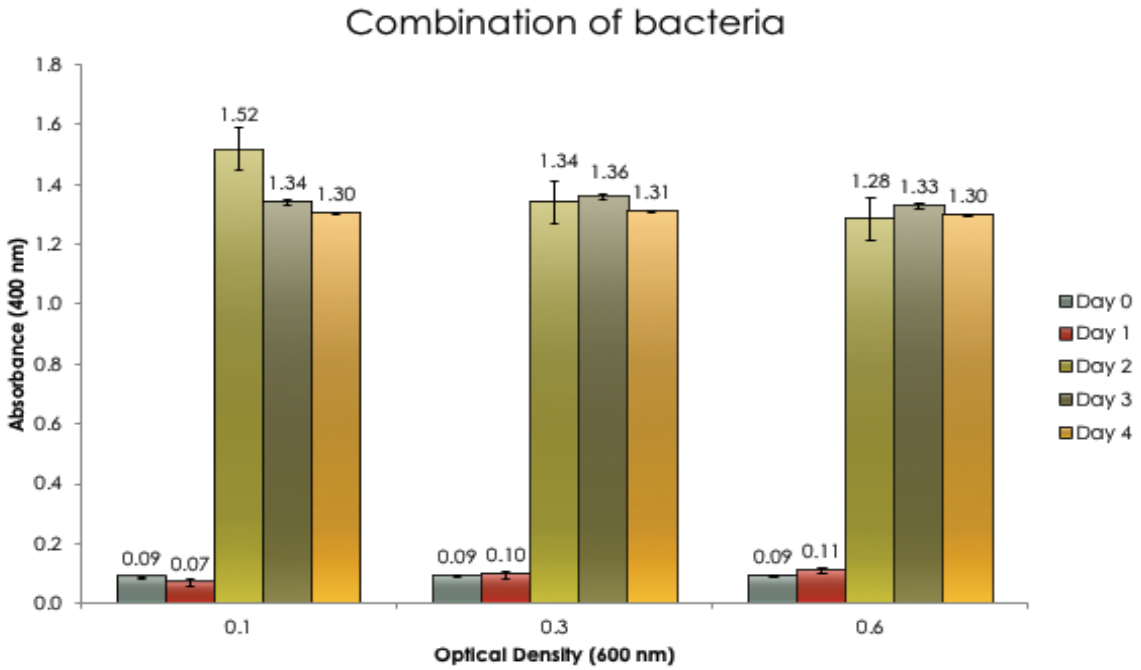


Figure 22. Protease assay for the combination of the three bacteria during the course of four days. The production of protease was measured under three optical densities (0.1, 0.3 and 0.6) for the microorganisms.

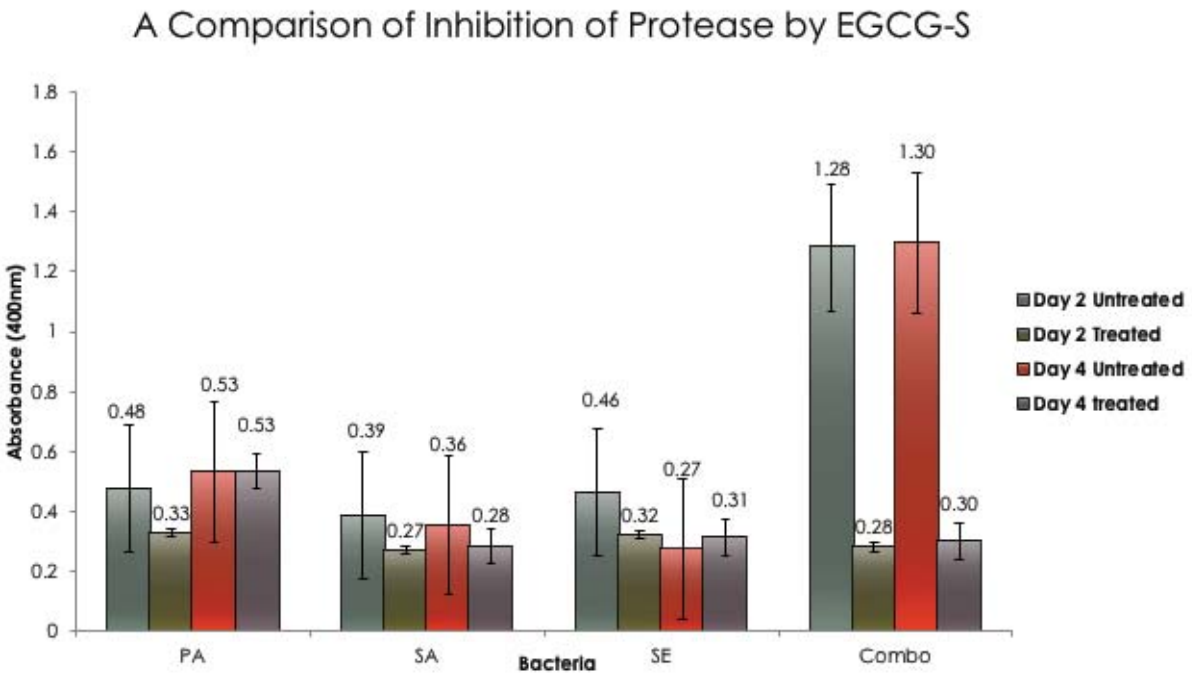


Figure 23. The use of EGCG-S to inhibit protease production. EGCG-S was used on bacteria grown for two and four days and compared against bacteria that were grown without it for the same number of days. The groups of bacteria were PA (*P. aeruginosa*), *S. epidermidis* (SE), *S. aureus* (SA) and Combo (*S. aureus*, *S. epidermidis* and *P. aeruginosa*). An optical density of 0.6 was used.

Table 2. The percent inhibition of protease production during Day 2 and day 4. Bacteria were treated with EGCG-S.

Bacteria	**Day 2**	**Day 4**
Pseudomonas aeruginosa	19.26 %	0 %
Staphylococcus aureus	30.10 %	20.20 %
Staphylococcus epidermis	30.44 %	0 %
Combination	78.03 %	76.79 %

The production of elastase was also measured for the three bacteria (Figures 24-27). Optical density did not seem to have a significant effect on the production of the enzyme. The amount of enzyme production stayed consistent throughout four days for *S. aureus*, *S. epidermidis* and *P. aeruginosa*. The combination of bacteria produced a considerable amount of elastase after day 1 of growth. EGCG-S was used to inhibit elastase production and the results were measured after two days (Figure 28). The greatest effect of percent inhibition was seen for the bacteria in combination with 77.09 %. (Table 3). The results suggested that the production of enzymes (virulent factors) are enhanced when the bacteria are in competition.

EGCG-S had an effect on inhibiting protease and elastase production. The next goal was to examine if it could work with the current treatment to prevent or reduce the production of virulent factors. Percent inhibition for W, WBP, and WBPT for *P. aeruginosa* and a combination of all three bacteria are shown in Figure 29 and 30. The results showed EGCG-S having little to no difference from the other two groups (Wash & Wash+PB). EGCG-S was seen to work better by itself when not combined with wash or antibiotics. Further studies would have to be done to determine why EGCG-S does not have an inhibitory effect when combined with the current surgical wash and antibiotics.

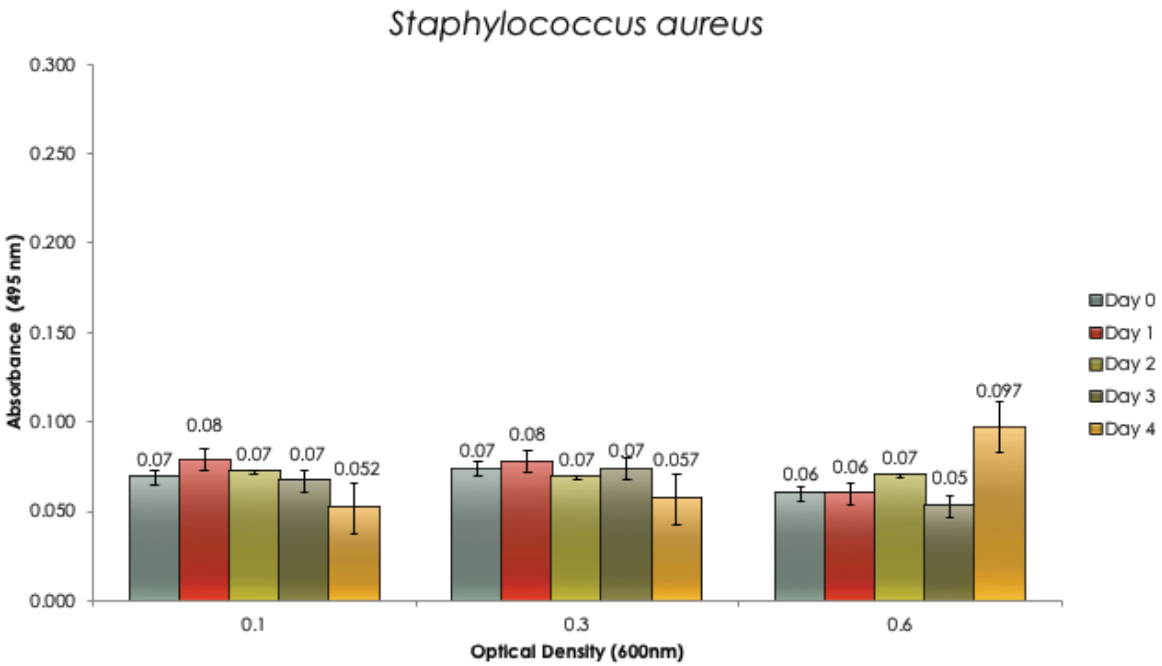


Figure 24. Elastase Assay for *S. aureus* during the course of four days. The production of elastase was measured under three optical densities (0.1, 0.3 and 0.6) for the microorganism.

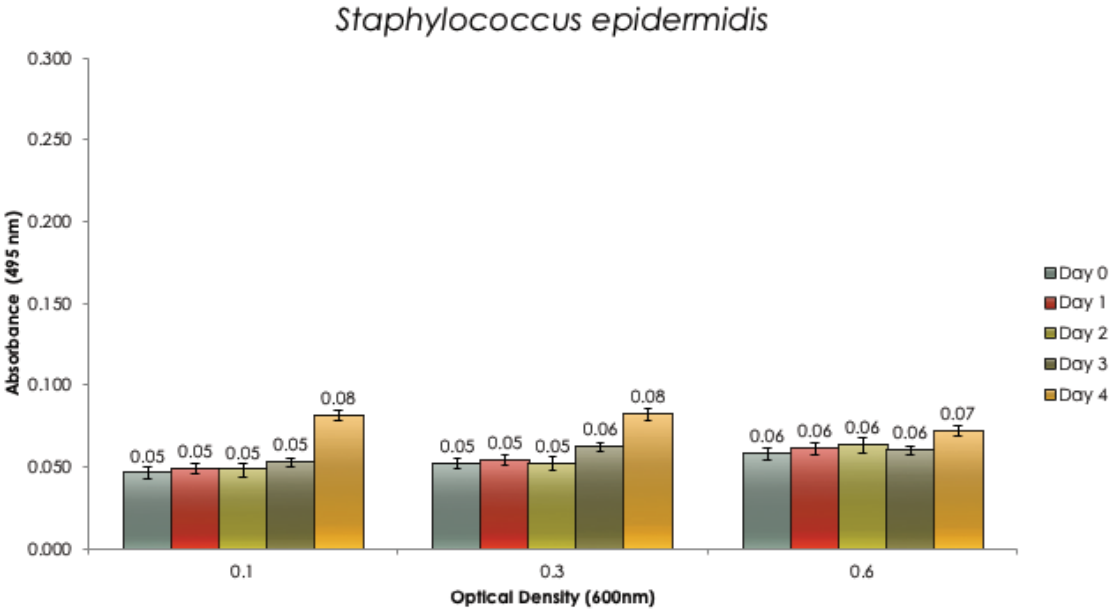


Figure 25. Elastase Assay for *S. epidermidis* during the course of four days. The production of elastase was measured under three optical densities (0.1, 0.3 and 0.6) for the microorganism.

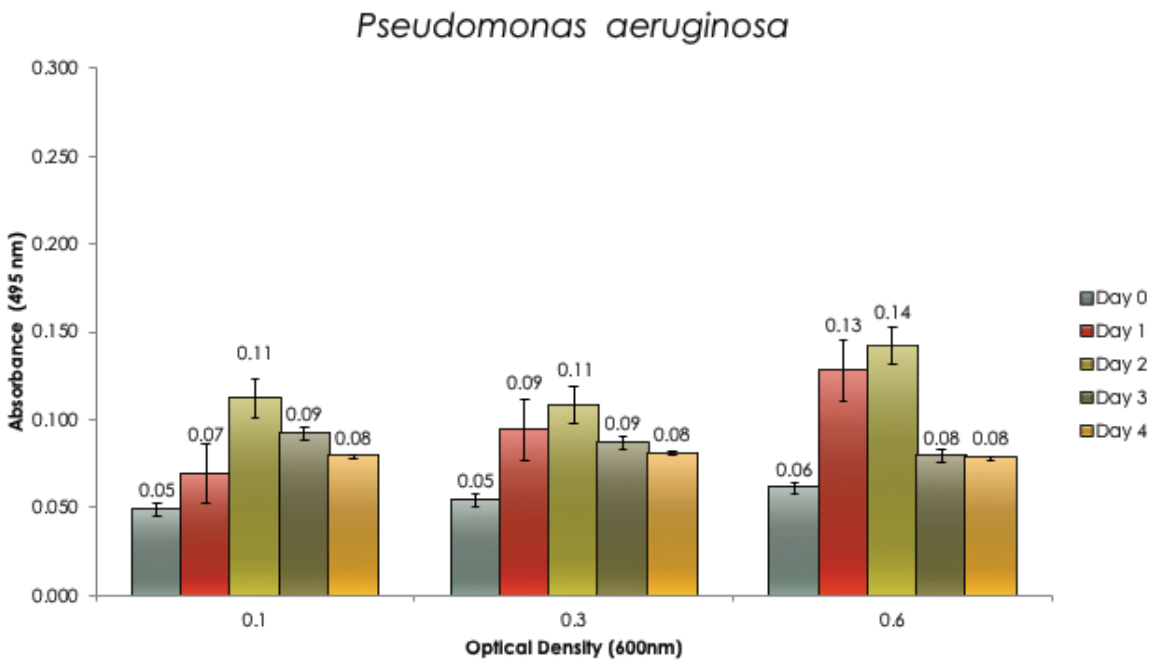


Figure 26. Elastase Assay for *P. aeruginosa* during the course of four days. The production of elastase was measured under three optical densities (0.1, 0.3 and 0.6) for the microorganism.

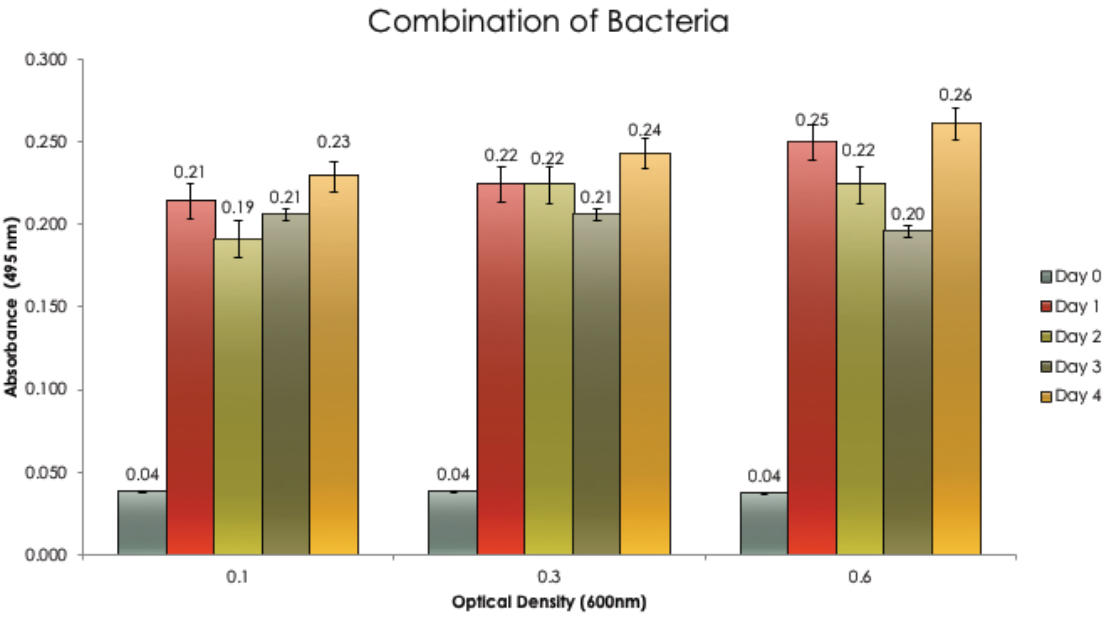


Figure 27. Elastase assay for the combination of *S. aureus, S. epidermidis* and *P. aeruginosa* the course of four days. The production of elastase was measured under three optical densities (0.1, 0.3 and 0.6) for the microorganism.

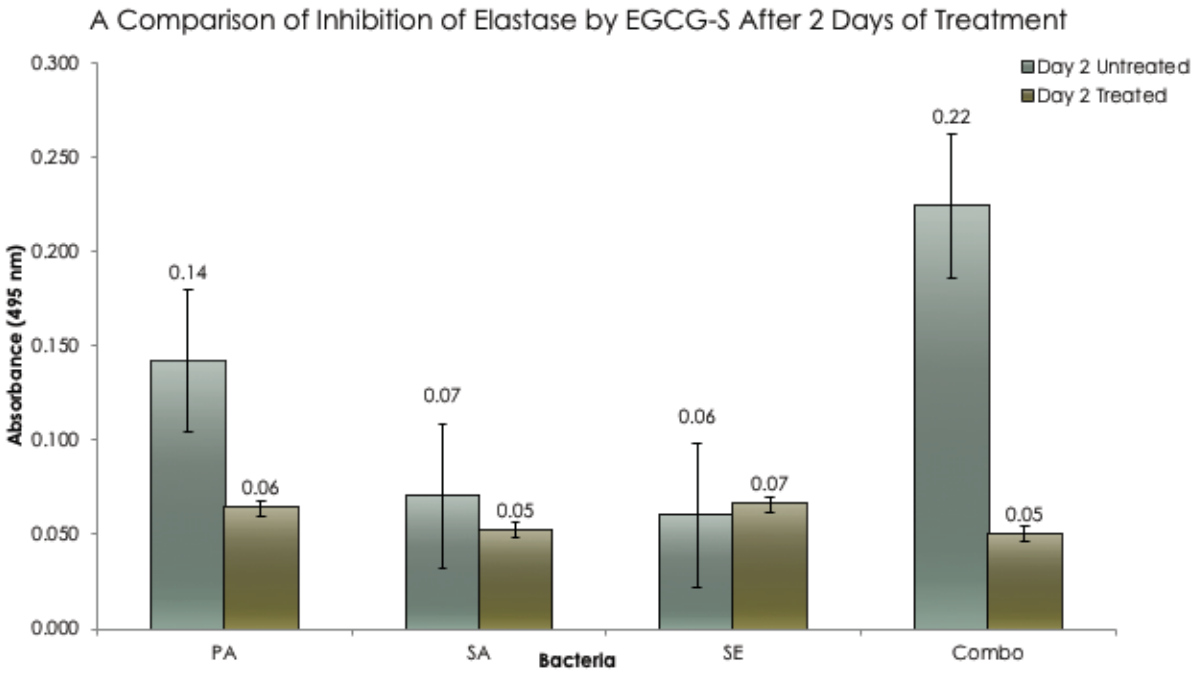


Figure 28. Inhibition of Elastase by EGCG-S after two days of growth. EGCG-S was used on bacteria grown for two and four days and compared against bacteria that were grown without it for the same number of days. The groups of bacteria were PA (*P. aeruginosa*), SE *(S. epidermidis)* SA *(S. aureus)* and Combo (*S. aureus*, *S. epidermidis* and *P. aeruginosa*). An optical density of 0.6 was used.

Table 3. Percent of inhibition of Elastase production during Day 2 of treatment with EGCG-S

Bacteria	**Day 2**
Pseudomonas aeruginosa	54.87 %
Staphylococcus aureus	26.16 %
Staphylococcus epidermidis	0 %
Combination	77.09 %

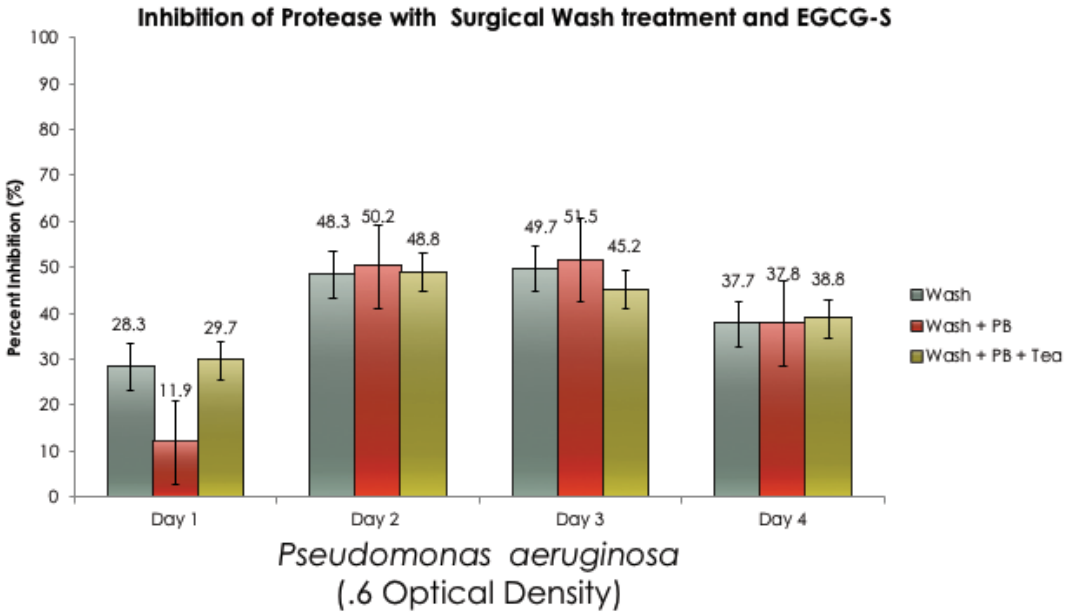


Figure 29. Percent Inhibition of protease for *P. aeruginosa* when treated with current treatment for infection and with the addition of EGCG-S. Optical density for the microorganism was prepared at 0.6. The bacteria were grown and treated for 1-4 days.

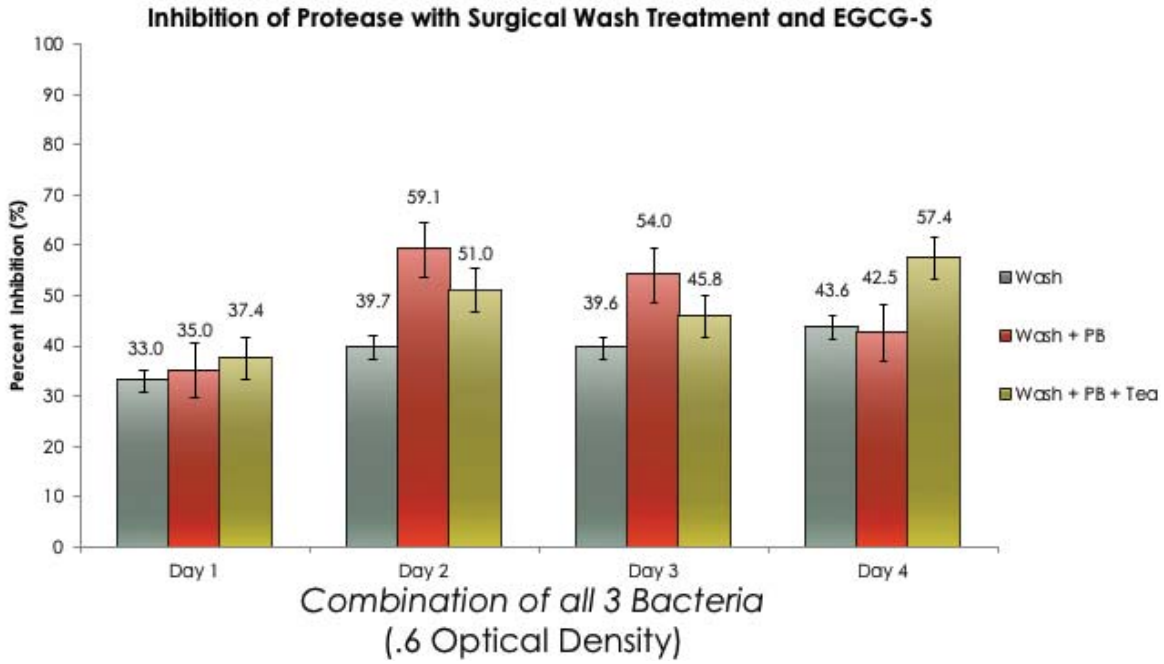


Figure 30. Percent Inhibition of protease production for the combination of three bacteria when treated with current treatment for joint infection and with the addition of EGCG-S. Optical density for the microorganisms were prepared at 0.6. The bacteria were grown and treated for 1-4 days.

Conclusions

Previous studies established 250 μg /ml EGCG-S as being the minimum inhibitory concentration for working against *S. aureus*, *S. epidermidis* an d *P.aeruginosa*. It was also established that EGCG-S could be a used during irrigation after implant has been placed; to prevent infection from occurring. Data gathered from this study looks at methods in which EGCG-S can be used for infections that have occurred due to implant surgery.

A survey of the current prosthetic joint wash protocol was examined; EGCG-S was used in combination to determine whether it could enhance the outcome. EGCG-S with current wash and antibiotics were shown to have some inhibitory effects for colony forming units. In treating a large population of *S.aureus* and *S. epidermidis*; 5 minutes was shown to be the start of treatment that would have the most beneficial effect. Other studies showed that for a serial diluted population of *S. aureus, S.epidermidis*, *P.aeruginosa* and a combination of all three ECGG-S had the most effect at the 30 minutes and 1 hour.

For inhibition of biofilm EGCG-S was shown to be just as effective when combined with wash and antibiotics for *S.aureus* and *S.epidermidis*. EGCG-S had a slight advantage in targeting biofilm caused by *P.aeruginosa*. Protease and elastase enzymes were seen to be produced predominantly when all three bacteria were existing together. EGCG-S worked to a greater extent for the combination of all three bacteria when it was used alone and not combined with the current treatment.

This study shows EGCG-S as having the possibility of improving the current treatment method by targeting acute infections formed by planktonic cells and providing an alternative method for chronic infections caused by biofilms.

Future Studies

The mechanism of action for how EGCG-S targets bacterial cells and biofilm will need to be further understood. The different genes detected for specific species as being responsible for biofilm and the pathogenicity of the bacteria will have to be further analyzed by PCR and/or qPCR to determine if EGCG-S plays a role in down-regulating the expression.

EGCG-S with the use of a single antibiotic would also have to be further examined to determine whether the same or greater effects can be observed when the antibiotics are not in combination. Different combinations of antibiotics will also be studied to determine the minimum inhibitory concentration that can be used with EGCG-S. Once this is established an examination of infected tissue culture and implant material will be studied.

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Appendix A | Nanodrop Readings

DNA Extraction	260/280	260/230	ng/ µl
Staphylococcus aureus	**1.99**	**1.01**	**816.1**
Staphylococcus epidermidis	**1.90**	**1.21**	**1042.8**
Pseudomonas aeruginosa	**1.86**	**0.97**	**764.5**

Appendix A. Nanodrop readings. DNA was extracted from *S. aureus*, *S. epidermidis* and *P. aeruginosa*. Column two shows the ratio of DNA to RNA (260/280). Column three ratio of DNA to impurities (260/230) and Column four has the concentration of DNA in ng/ µl. The DNA was stored and used with species specific primers to check for the presence of certain genes.

Appendix B | *Staphylococcus aureus* primers for sequence analysis

Staphylococcus aureus				
Gene	**Sequence 5'-3'**	**Tm (°C)**	**PCR/qPCR**	**Function**
16s_rRNA_F_SA	TGTCGTGAGATGTTGGG	57.2	qPCR	16s RNA
16s_rRNA_R_SA	CGATTCCAGCTTCATGT	58.4	qPCR	16s RNA
clfA_F	AGAAACGCCGGTAACT	54.2	qPCR	Clumping factor
clfA_R	CTCTCA TTCTAGGCGCAC	59.9	qPCR	Clumping factor
clfB_F	GCGCATTGGAAATCGT	54.2	qPCR	Clumping factor
clfB_R	AGAGCCAGCTTCAACA	54.2	qPCR	Clumping factor
FnbA_F	ATGA TCGTTGTTGGGATG	55.3	PCR	Fibronectin binding protein
FnbA_R	GCAGTTTGTGGTGCTTGT	57.6	PCR	Fibronectin binding protein
FnbB_F	ACAAGTAATGGTGGGTAC	55.3	PCR	Fibronectin binding protein
FnbB_R	AATAAGGATAGTATGGGT	50.1	PCR	Fibronectin binding protein
IcaA_F	TTTGGGTGTCTTCACTCTAT	58.7	PCR	Slime production
IcaA_R	CCTAGTAATACTTCGTGTCCC	60.6	PCR	Slime production
IcaR_F	ATCTAATACGCCTGAGGA	55.3	PCR	Transcriptional repressor
IcaR_R	TTCTTCCACTGCTCCA	54.8	PCR	Transcriptional repressor
spa_F	TTTGTCAGCAGTAGTGCC	57.6	PCR	Allows SA to invade host immune system
spa_R	ACGA TCCTTCAGTGAGC	57.2	PCR	Allows SA to invade host

				immune system

Appendix B. Primers for *S.aureus*. Forward primer is abbreviated with F and reverse with R. Annealing temperature and the gene function are also provided.

Appendix C | *Staphylococcus epidermidis* primers for sequence analysis

Staphylococcus epidermidis				
Gene	**Sequence**	**Tm (°C)**	**PCR/qPCR**	**Function**
Aap1_F	ATATGGGCAAACGTAGACAAGTC	62	PCR	Subunits for intracellular lipid kinases
Aap1_R	AGTTGGCGGTATATCTATTGTA	64.6	PCR	Subunits for intracellular lipid kinases
Aap2_F	GAAGCACCGAATGTTCCAACTATC	62.9	PCR	Subunits for intracellular lipid kinases
Aap2_R	TGGTTCTGCTTTTGTTGGACCATAC	62.9	PCR	Subunits for intracellular lipid kinases
icaADB_F	TTATCAATGCCGCAGTTGTC	70.8	PCR	Biofilm producing ability
icaADB_R	GTTTAACGCGAGTGCGCTAT	60.4	PCR	Biofilm producing ability

Appendix C. Primers for *S. epidermidis*. Forward primer is abbreviated with F and reverse with R. Annealing temperature and the gene function are also provided.

Appendix D | *Pseudomonas aeruginosa* primers for sequence analysis

Pseudomonas aeruginosa				
Gene	**Sequence**	**Tm (°C)**	**PCR/qPCR**	**Function**
16s_rRNA_PA_F	CAAAACTACTGAGCTAGAGTACG	61	PCR	16sRNA
16s_rRNA_PA_R	GCCACTGGTGTTCCTTCCTA	62.4	PCR	16sRNA
OprL_F	CCAACAGCGGTGCCGTTGA	64.5	qPCR	outer membrane integrity
OprL_R	GCGATATTGTACTCGCGGGT	62.4	qPCR	outer membrane integrity
ToxA_F	GGAGCGCAACTATCCCACT	62.3	qPCR	Blocks protein synthesis
ToxA_R	TGGTAGCCGACGAACACATA	60.4	qPCR	Blocks protein synthesis
PA1169_F	GCGGAACAACAGGGTCTA T	60.4	qPCR	Protein coding lipoxygenase
PA1169_R	CCTTTGACATTGCCGAAGTT	58.4	qPCR	Protein coding lipoxygenase

Appendix D. Primers for *P.aeruginosa.* Forward primer is abbreviated with F and reverse with R. Annealing temperature and the gene function are also provided.

Appendix E | Polymerase chain reaction parameters

Stage	**Tm (°C)**	**Time**	**Number of Cycles**
Denaturation	95	1 minutes	1
Denaturation	95	20 seconds	30
Annealing	Varies	20 seconds	30
Extension	72	1 min	30
Extension and polishing	72	7 minutes	1
Storage	4	∞	N/A

Appendix E. PCR Parameters. These parameters were used with the primers for *S.aureus*, *S.epidermidis* and *P.aeruginosa*. Annealing temperature varied according to the Tm listed for the primer.

Appendix F | Gel electrophoresis and Sequence protocol

Gel Electrophoresis

A 1% gel agarose was made by measuring out 0.4 g of agarose and dissolving it in 40 ml of 1x Tris-Acetate-EDTA (TAE) buffer. A 2% gel was prepared by using 0.8 g of agarose instead of 0.4 g. After the agarose was added to the TAE, it was heated in a microwave for a few seconds until the agarose fully dissolved. 4 µl of SYBR safe was added to the solution and swirled gently. This mixture was used to cast a gel, the gel tray was first placed in the gel box at a 90-degree angle forming a seal. A comb was added to the front end of the gel tray and one in the middle end of the tray if more than 10 samples were being used. Once the solution was cool to the touch it was poured into the tray. The gel was allowed to solidify (approx. 20 minutes). TAE buffer was then poured on top of the gel until it was fully submerged. The comb/combs were carefully removed. Samples were prepared for well loading by pipetting 10 µl of PCR product into a clean new tube. 2 µl of loading dye was added to the 10 µl of PCR product and mixed. The full 12 µl was loaded into a well. The gel was then ran at 115 volts for 30 minutes. Visualization of the gel took place under a blue transilluminator.

Sequencing and Blast

A sample of the PCR product was sent for sequencing if a band was observed on the gel. The laboratory and equipment technician at Montclair State University developed the sequence. Once sequences were acquired a BLAST search was done on the nucleotides. https://blast.ncbi.nlm.nih.gov/Blast.cgi.

Appendix G | Gel electrophoresis, Sequence and BLAST results

A.

B.

Well #:	Primer:	Tm (°C)
1	Hi-Lo Ladder	
2	Control	
3	16s RNA (SE)	
4	16s RNA (SA)	57.4 (F) 58.4 (R)
5	16s RNA (PA)	61.0 62.4
6	FnbA (SA)	55.3 57.6
7	oprL (PA)	64.5 62.4
8	icaADB (SE)	70.8 60.4

C.

16s RNA (SE) Primer 3F - 127bps

GCCCCATTCCGCTCGGCATGGGGGTGGTCCAGTTTCGAATGCACCCTCCGCG
GGGGAGCCGTGGGTGTCCGGACAATAATGAGACCCGAGCCGCCGGCGATAA
CGCGAACCCTGAGCGGGGACCCCA

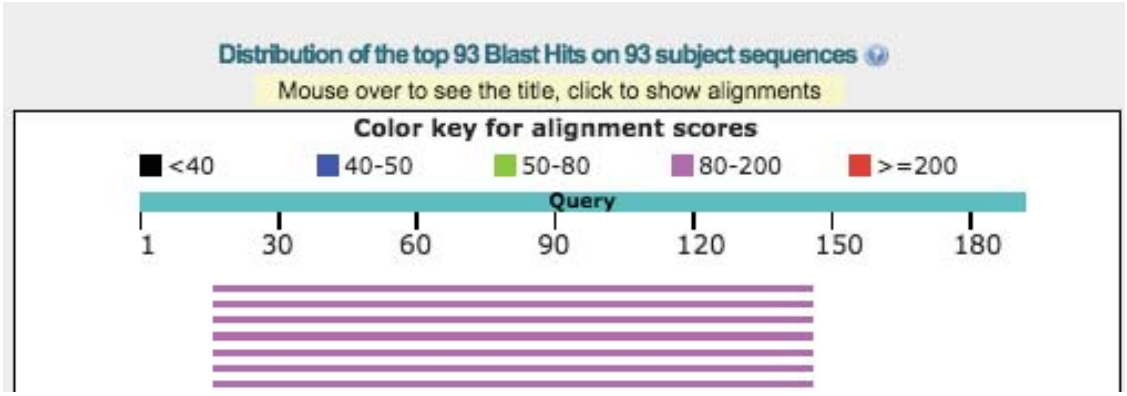
16s RNA (SE) Primer 3F- Blast Search

No significant similarity found

FnbA (SA) Primer 6F - 191bps

GGGGTGCACGTCCAGGAACTACAGTAGAGAATGGGATTCAGCTACTGATAAT
AAAGTAAGCGAAACACAAACAACTACACTAACGTTAATACTATAGATGAAAC
ACAATCATACAGCGCAACAGCCAAAGAACTCCCGTCACACGGTAGAACTGA
GAGCCCGCATTATTTGGCCCCCCCACCGCAGATAAA

FnbA (SA) Primer 6F - Blast Search



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Staphylococcus aureus strain 13420 chromosome	187	187	67%	9e-44	93%	CP021141.1
Staphylococcus aureus strain 0201753-2 genome	187	187	67%	9e-44	93%	CP021352.1
Staphylococcus aureus strain AR_0470 chromosome, complete genome	187	187	67%	9e-44	93%	CP029653.1
Staphylococcus aureus strain CFSAN007896 chromosome, complete genome	187	187	67%	9e-44	93%	CP020467.1
Staphylococcus aureus strain CFSAN018749 chromosome, complete genome	187	187	67%	9e-44	93%	CP028190.1
Staphylococcus aureus strain NRS143 chromosome, complete genome	187	187	67%	9e-44	93%	CP026071.1
Staphylococcus aureus strain NRS484 chromosome, complete genome	187	187	67%	9e-44	93%	CP026066.1
Staphylococcus aureus strain CFSAN007847 chromosome, complete genome	187	187	67%	9e-44	93%	CP017684.1
Staphylococcus aureus strain Seattle 1945 isolate G478, complete genome	187	187	67%	9e-44	93%	CP021907.1
Staphylococcus aureus strain Seattle 1945 isolate G477, complete genome	187	187	67%	9e-44	93%	CP021905.1
Staphylococcus aureus strain SJTUF_J27, complete genome	187	187	67%	9e-44	93%	CP019117.1
Staphylococcus aureus subsp. aureus strain FORC_001, complete genome	187	187	67%	9e-44	93%	CP009554.1
Staphylococcus aureus genome assembly Staphylococcus_aureus_ILRI_Eymole1/1, chromosome : I	187	187	67%	9e-44	93%	LN626917.1
Staphylococcus aureus subsp. aureus strain ATCC 25923, complete genome	187	187	67%	9e-44	93%	CP009361.1
Staphylococcus aureus subsp. aureus 55/2053, complete genome	187	187	67%	9e-44	93%	CP002388.1
Staphylococcus aureus subsp. aureus TCH60, complete genome	187	187	67%	9e-44	93%	CP002110.1
Staphylococcus aureus subsp. aureus ED133, complete genome	187	187	67%	9e-44	93%	CP001996.1
Staphylococcus aureus subsp. aureus strain ATCC 25923 fibronectin binding protein A (fnbA) gene, partial cds	187	187	67%	9e-44	93%	EU195388.1

OprL (PA) Primer 7F- 133bps

CAATTCGGGTTTCTTGCTGATTCTGCAGCACGTCGCGCGTCTTTATAGTCAGG
TTGTCATATTGGGCAGAGTCGGCGTAATCAGGTTGTCCAGCGAACTTCGCGA
GGTCCTATGTTCAGGTGTTGTTCCGCA

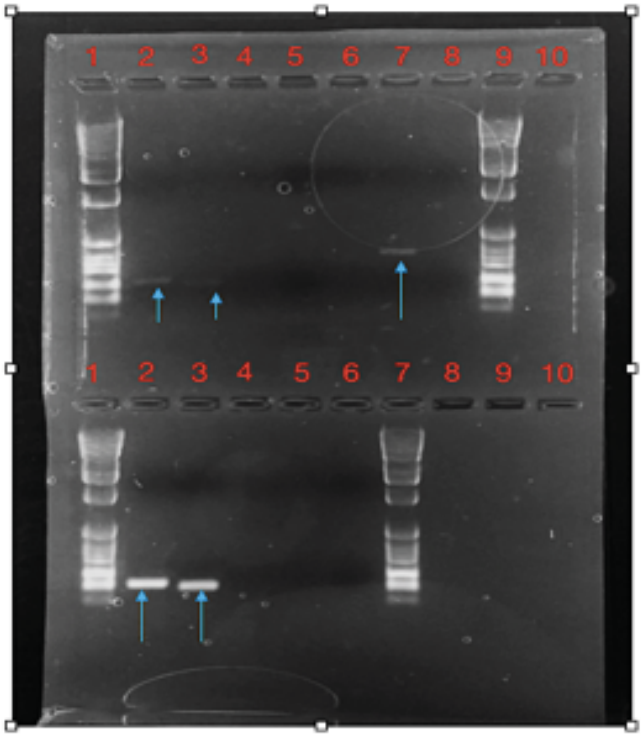
OprL (PA) Primer 7F- Blast Search

No significant similarity found

Appendix G. Gel electrophoresis, sequence and BLAST search (a). A 1% agarose gel was made for the primers used on SA (*S. aureus*), SE (*S. epidermidis)* and PA (*P. aeruginosa)*. A band is shown if the gene is present. The first row contains the ladder. A molecular weight chart is shown to the left of the first well to depict a clearer image of the molecular weight. **(b).** The table corresponds to the wells on the gel and what is in each one. Primers specific to *S. aureus*, *S. epidermidis* and *P. aeruginosa* were obtained. A 1:10 dilution was made for the primers. Primers highlighted in red represent the gene being detected in the microorganism. **(c)** One primer for each microorganism was chosen for sequencing, afterwards a blast search was done.

Appendix H | Gel electrophoresis, sequence and BLAST results

A.



B.

Top:

Well #:	Primer:	Tm (°C)
1	Hi-Lo Ladder	--
2	icaA (SA)	58.7(F)/60.6(R)
3	icaR (SA)	55.3 / 54.8
4	c1fA (SA)	54.2 / 59.9
5	Control	--
6	c1fB (SA)	54.2 / 54.2
7	spa (SA)	57.6 / 57.2
8	FnbB (SA)	55.3 / 50.1
9	Hi-Lo Ladder	--

Bottom:

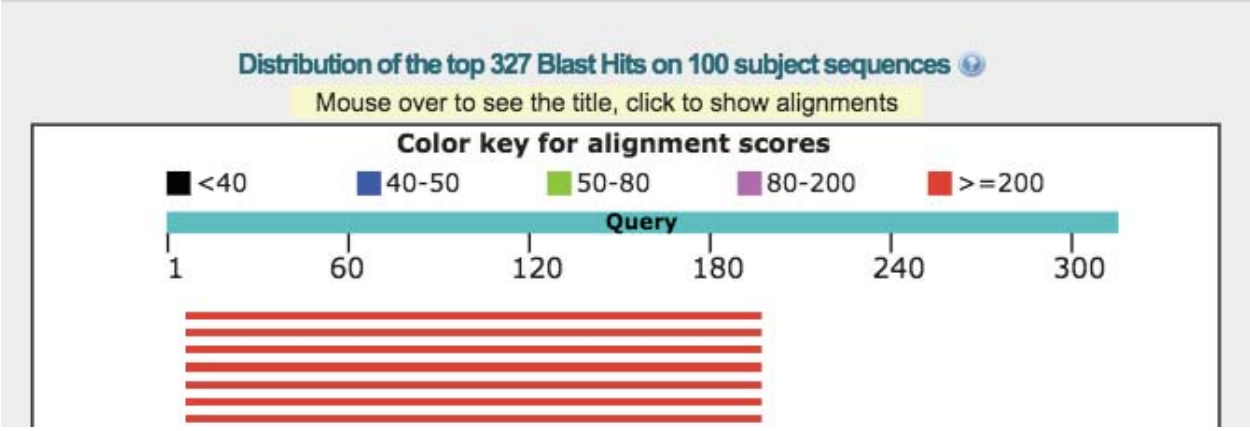
Well #:	Primer:	Tm (°C) [F / R]
1	Hi-Lo Ladder	--
2	PA1169 (PA)	60.4 / 58.4
3	ToxA (PA)	62.3 / 60.4
4	Control	--
5	Aap1 (SE)	62.0 / 64.6
6	icaADB (SE)	70.8 / 60.4
7	Hi-Lo Ladder	--

C.

SpA (SA) Primer 7F- 298 bps

AGACCAGTACTGTATCACACGCTGACGACATGTACTCCGTTGCCGTCTTCTTT
ACCAGGCTTGTTGCATCTTCTTTACCAGGCTTGTTGCCGTCTTCTTTACCAGGT
TTGTTGCCATCTTCTTTGCCAGGTTTTTTGTTGTCTTCTTTACCAGGTTTGTTGC
CGTCTTCTTTGCCCGGCAAGATGTTGACTTCTTTACAAGAGAGGTAGCGCCCG
TAAGAACTGGAATAGCCTGTTACAGATGCGCTGACTTCTCTGTTGACCTGAGC
TCGTTAATTTTTGCTCGCAAACTTCTTGCTGACAAAAGACGTATC

SpA (SA) Primer 7F Blast Search



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Staphylococcus aureus strain 13420 chromosome	187	187	67%	9e-44	93%	CP021141.1
Staphylococcus aureus strain 0201753-2 genome	187	187	67%	9e-44	93%	CP021352.1
Staphylococcus aureus strain AR_0470 chromosome, complete genome	187	187	67%	9e-44	93%	CP029653.1
Staphylococcus aureus strain CFSAN007896 chromosome, complete genome	187	187	67%	9e-44	93%	CP020467.1
Staphylococcus aureus strain CFSAN018749 chromosome, complete genome	187	187	67%	9e-44	93%	CP028190.1
Staphylococcus aureus strain NRS143 chromosome, complete genome	187	187	67%	9e-44	93%	CP026071.1
Staphylococcus aureus strain NRS484 chromosome, complete genome	187	187	67%	9e-44	93%	CP026066.1
Staphylococcus aureus strain CFSAN007847 chromosome, complete genome	187	187	67%	9e-44	93%	CP017684.1
Staphylococcus aureus strain Seattle 1945 isolate G478, complete genome	187	187	67%	9e-44	93%	CP021907.1
Staphylococcus aureus strain Seattle 1945 isolate G477, complete genome	187	187	67%	9e-44	93%	CP021905.1
Staphylococcus aureus strain SJTUF_J27, complete genome	187	187	67%	9e-44	93%	CP019117.1
Staphylococcus aureus subsp. aureus strain FORC_001, complete genome	187	187	67%	9e-44	93%	CP009554.1
Staphylococcus aureus genome assembly Staphylococcus_aureus_ILRI_Eymole1/1, chromosome : I	187	187	67%	9e-44	93%	LN626917.1
Staphylococcus aureus subsp. aureus strain ATCC 25923, complete genome	187	187	67%	9e-44	93%	CP009361.1
Staphylococcus aureus subsp. aureus 55/2053, complete genome	187	187	67%	9e-44	93%	CP002388.1
Staphylococcus aureus subsp. aureus TCH60, complete genome	187	187	67%	9e-44	93%	CP002110.1
Staphylococcus aureus subsp. aureus ED133, complete genome	187	187	67%	9e-44	93%	CP001996.1
Staphylococcus aureus subsp. aureus strain ATCC 25923 fibronectin binding protein A (fnbA) gene, partial cds	187	187	67%	9e-44	93%	EU195388.1

ToxA (PA) Primer 3F (bottom row)- 113 bps

GCATTGGGGCAGGGTTCTATTCAGCCTTTCGCATCGTGCAGCAGACTGGACG
GTGGACGACTGCTGCGCCGTGGCAGTTTCGCACGTGGAATGCGCTATACGTT
CAGCGGGCT

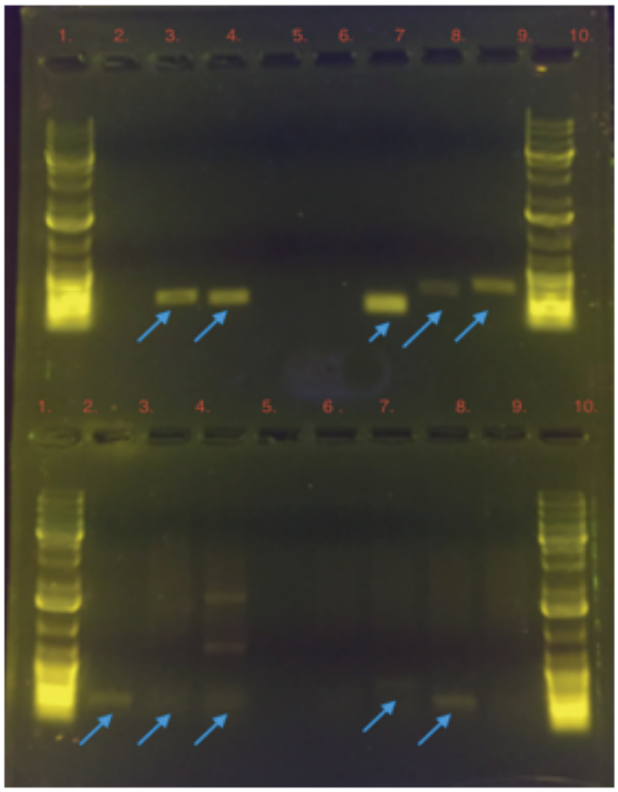
ToxA (PA) Primer 3F (bottom row)- Blast Search

No significant similarity found

Appendix H. Gel electrophoresis. (a). A 2% agarose gel was made for the primers used on SA (*S. aureus*), SE (*S. epidermidis)* and PA (*P. aeruginosa)*. A band (above blue arrow) is shown if the gene is present. The first row contains the ladder. **(b).** The table corresponds to the wells on the gel and what is in each one. Primers specific to *S. aureus*, *S. epidermidis* and *P. aeruginosa* were used. A 1:10 dilution was made for the primers. Primers in red represent the gene being detected in the microorganism. **(c)** One primer that worked was chosen for each microorganism and sequenced. A blast search was done with the sequence data.

Appendix I | Gel electrophoresis, Sequence and BLAST results for Crossmatch of primers

A.



B.

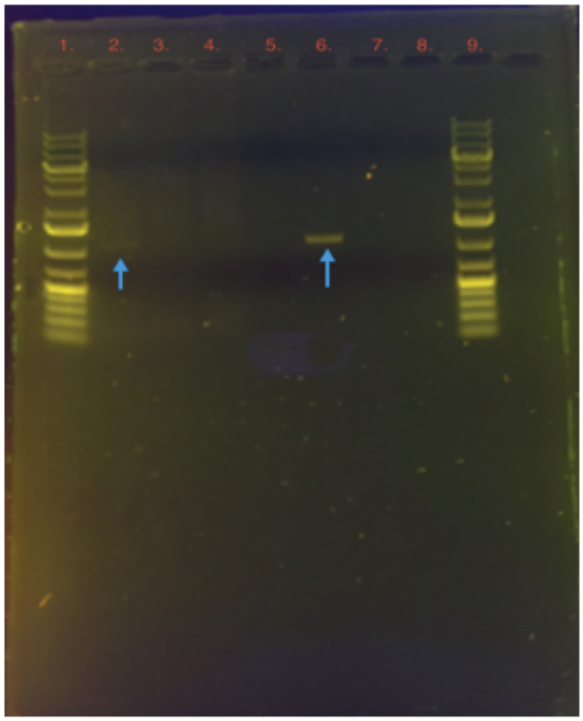
Top

Well #:	Primer:	Tm (°C) [F/R]
1	Hi-Lo Ladder	—
2	fnbB (SA)- SE	55.3 / 50.1
3	FnbA (SA)-SE	55.3 /57.6
4	c1fB (SA)-SE	54.2 / 54.2
5	Water	Control
6	icaR (SA)-SE	55.3 / 54.8
7	ToxA (PA)-SE	62.3 / 60.4
8	c1fA (SA)-SE	54.2 / 59.9
9	spa (SA)- SE	57.6 / 57.2
10	Hi-Lo Ladder	—

Bottom

Well #:	Primer:	Tm (°C) [F/R]
1	Hi-Lo Ladder	--
2	icaA (SA)-SE	58.7 / 60.6
3	c1fA (SA)-PA	54.2 / 59.9
4	c1fB (SA)-PA	54.2 / 54.2
5	Control	—
6	fnbB (SA)-PA	55.3 / 50.1
7	spa (SA)- PA	57.6 / 57.2
8	fnbA(SA)- PA	55.3 / 57.6
9	icaA(SA)-PA	58.7 / 60.6
10	Hi-Lo Ladder	--

C.



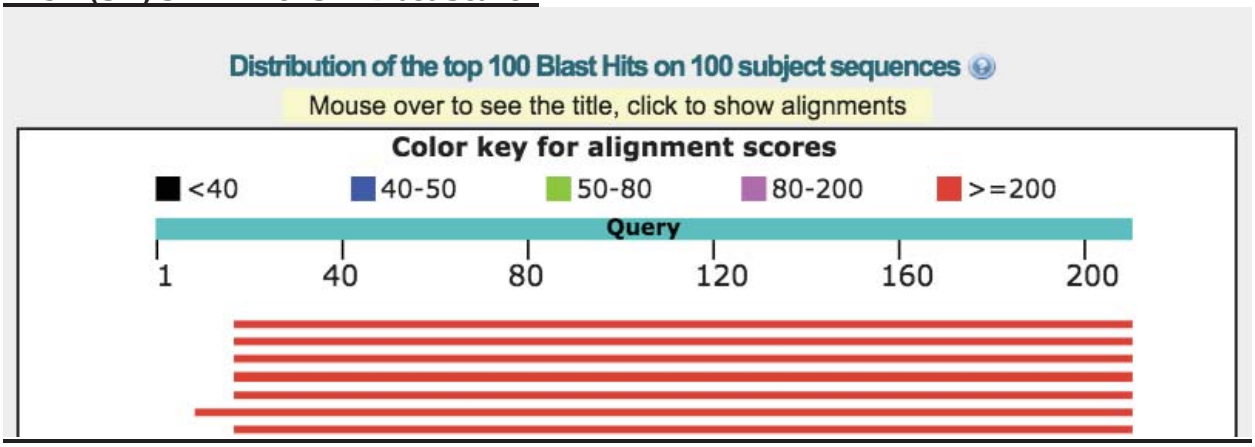
D.

Well #:	Primer:	Tm (°C) [F/R]
1	Hi-Lo Ladder	--
2	Apa1(PA)- SE	62.0 / 64.6
3	icaR(PA)-SA	55.3 / 54.8
4	icaADB (PA)	70.8 / 60.4
5	Water	--
6	Apa1(SA)-SE	62.0 / 64.6
7	Tox(SA)-PA	62.3 / 60.4
8	icaADB (SA)- SE	70.8 / 60.4
9	Ladder	--

FnbA (SA) SE Primer 3F- 210bps

TTGATCCAAATCAGCACAAGACACTACAGTAGAAGAAATGGGAATTCCAGCT
ACTGATAATAAAGTAAGCGAAACACAAACAACTACAACTAACGTTAATACTA
TAGATGAAACACAATCATACAGCGCAACAGCAACAGAACAACCGTCAAACG
CAACACAAGTAACAACTGAAGAAGCACCAAAAGCAGTACAAGCCCCACAAA
CTGC

FnbA (SA) SE Primer 3F -Blast Search



Sequences producing significant alignments:

Select: All None Selected:0

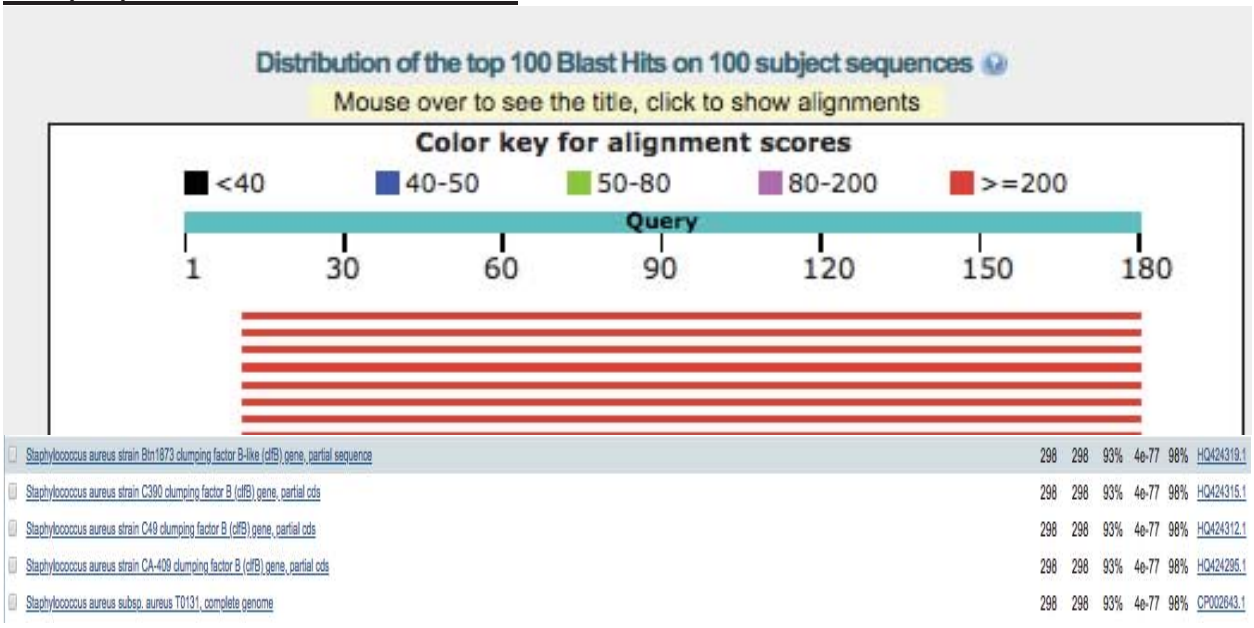
Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Staphylococcus aureus strain 13420 chromosome	335	335	91%	4e-88	98%	CP021141.1
Staphylococcus aureus strain 0201753-2 genome	335	335	91%	4e-88	98%	CP021352.1
Staphylococcus aureus strain AR_0470 chromosome, complete genome	335	335	91%	4e-88	98%	CP029653.1
Staphylococcus aureus strain CFSAN007896 chromosome, complete genome	335	335	91%	4e-88	98%	CP020467.1
Staphylococcus aureus strain CFSAN018749 chromosome, complete genome	335	335	91%	4e-88	98%	CP028190.1
Staphylococcus aureus strain NRS143 chromosome, complete genome	335	335	95%	4e-88	97%	CP026071.1
Staphylococcus aureus strain NRS484 chromosome, complete genome	335	335	91%	4e-88	98%	CP026066.1
Staphylococcus aureus strain CFSAN007847 chromosome, complete genome	335	335	91%	4e-88	98%	CP017684.1
Staphylococcus aureus strain Seattle 1945 isolate G478, complete genome	335	335	91%	4e-88	98%	CP021907.1
Staphylococcus aureus strain Seattle 1945 isolate G477, complete genome	335	335	91%	4e-88	98%	CP021905.1
Staphylococcus aureus strain SJTUF_J27, complete genome	335	335	91%	4e-88	98%	CP019117.1
Staphylococcus aureus subsp. aureus strain FORC_001, complete genome	335	335	91%	4e-88	98%	CP009554.1
Staphylococcus aureus genome assembly Staphylococcus_aureus_ILRI_Eymole1/1, chromosome : I	335	335	91%	4e-88	98%	LN626917.1
Staphylococcus aureus subsp. aureus strain ATCC 25923, complete genome	335	335	91%	4e-88	98%	CP009361.1
Staphylococcus aureus subsp. aureus 55/2053, complete genome	335	335	91%	4e-88	98%	CP002388.1
Staphylococcus aureus subsp. aureus TCH60, complete genome	335	335	95%	4e-88	97%	CP002110.1
Staphylococcus aureus subsp. aureus ED133, complete genome	335	335	91%	4e-88	98%	CP001996.1
Staphylococcus aureus subsp. aureus strain ATCC 25923 fibronectin binding protein A (fnbA) gene, partial cds	335	335	95%	4e-88	97%	EU195388.1
Staphylococcus aureus partial fnbA gene for fibronectin binding protein A, strain 3011 ST-30	335	335	95%	4e-88	97%	AM749007.1

clfB (SA) SE Primer 4F -180bps

GTGTATCTATGTGAGGATTTTAGCTCACTGTTTGTTGCTATGTTATTAGCATCA
TTCGTTGTTTTATTATCTACTTGAGAATTTGCTTCTTGAGGAACAGTTTGATCT
TGCATTTTTGCAGCAGTTGCTTGATCTTTAATTGCCGTCGGTTGAGGTGTTTCA
TTTGATGAAGCTGGCTCT

clfB (SA) SE -Primer 4F-Blast Search

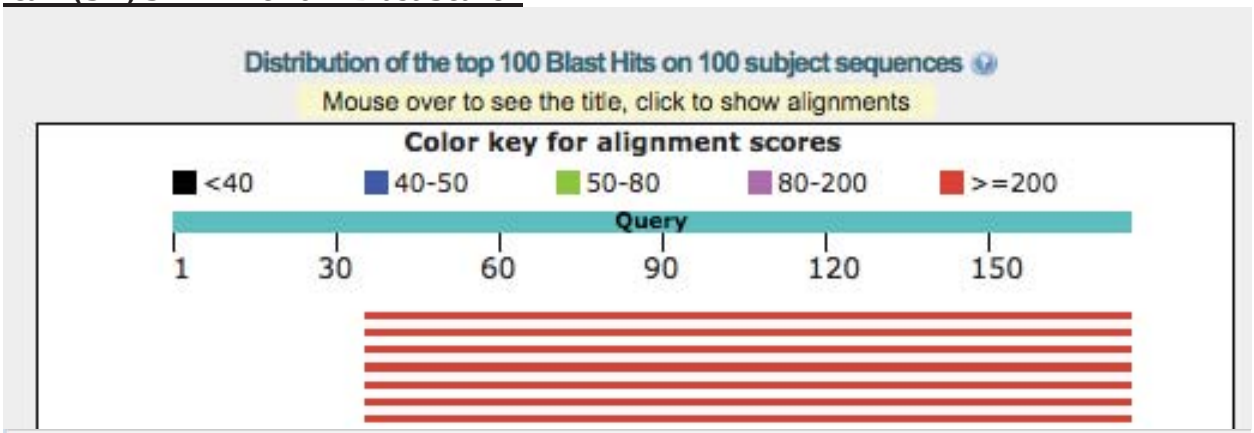


Staphylococcus aureus strain Btn1873 clumping factor B-like (clfB) gene, partial sequence	298	298	93%	4e-77	98%	HQ424319.1
Staphylococcus aureus strain C390 clumping factor B (clfB) gene, partial cds	298	298	93%	4e-77	98%	HQ424315.1
Staphylococcus aureus strain C49 clumping factor B (clfB) gene, partial cds	298	298	93%	4e-77	98%	HQ424312.1
Staphylococcus aureus strain CA-409 clumping factor B (clfB) gene, partial cds	298	298	93%	4e-77	98%	HQ424295.1
Staphylococcus aureus subsp. aureus T0131, complete genome	298	298	93%	4e-77	98%	CP002643.1

icaR (SA) SE Primer 6F -180bps

GAGAAGTTTGAAAATACAACTGTGGAGGGGCAGAAAATATTAAATTCTGAAG
TCTTCTTTTGTCATCTTTATTTTTGATTCATCATAGAGTTTGCTATCTCTTTACT
TAGTGGTTGATTTAAATCTTGTATTTATCCGTAAATATTTCCAGAAAATTCCTC
AGGCGTATTAGATA

icaR (SA) SE Primer 6F -Blast Search



Sequences producing significant alignments:

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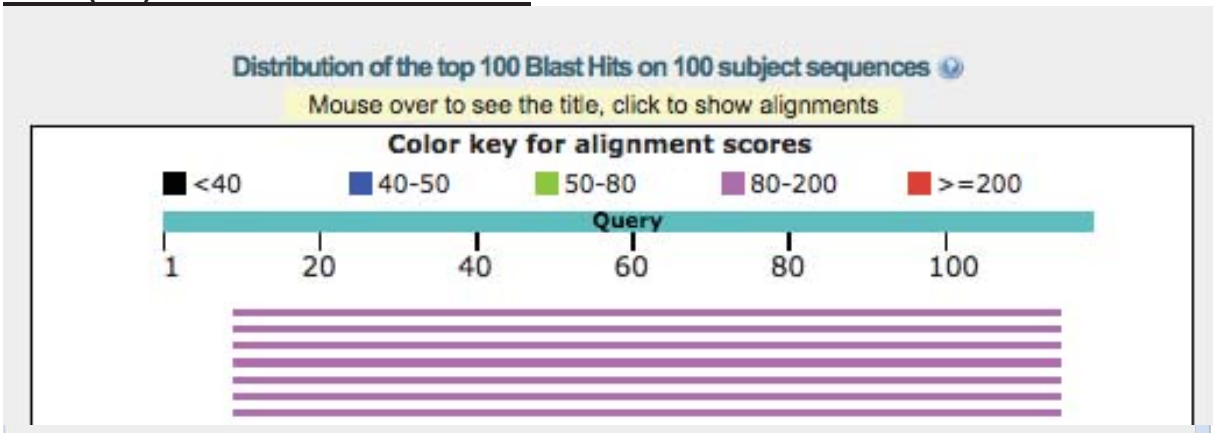
Alignments Download GenBank Graphics Distance tree of results

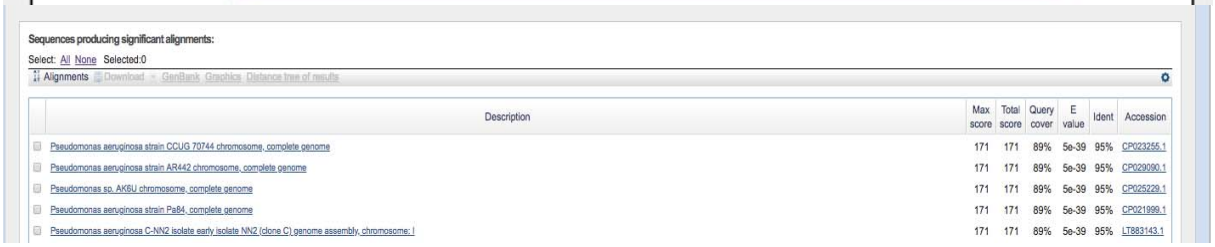
Description	Max score	Total score	Query cover	E value	Ident	Accession
Staphylococcus aureus strain 13420 chromosome	228	228	80%	5e-56	96%	CP021141.1
Staphylococcus aureus strain 0201753-2 genome	228	228	80%	5e-56	96%	CP021352.1
Staphylococcus aureus strain AR_0470 chromosome, complete genome	228	228	80%	5e-56	96%	CP029653.1
Staphylococcus aureus strain AR_0471 chromosome, complete genome	228	228	80%	5e-56	96%	CP029652.1
Staphylococcus aureus strain M92 chromosome, complete genome	228	228	80%	5e-56	96%	CP015447.2

ToxA (PA) SE Primer 7F -178bps

GCTATCGGCGCGGCGCCATCAGCTTCCAGCACCGCGCACGCAGACTGGACGG
TGGAGCGGCTGCTCCAGGCGCACCGCCAACTGGAGGAGCGCGGCTATGTGTT
CGTCGGCTACCTGGG

ToxA (PA) SE Primer 7F-Blast Search





Sequences producing significant alignments:

Select: All None Selected:0

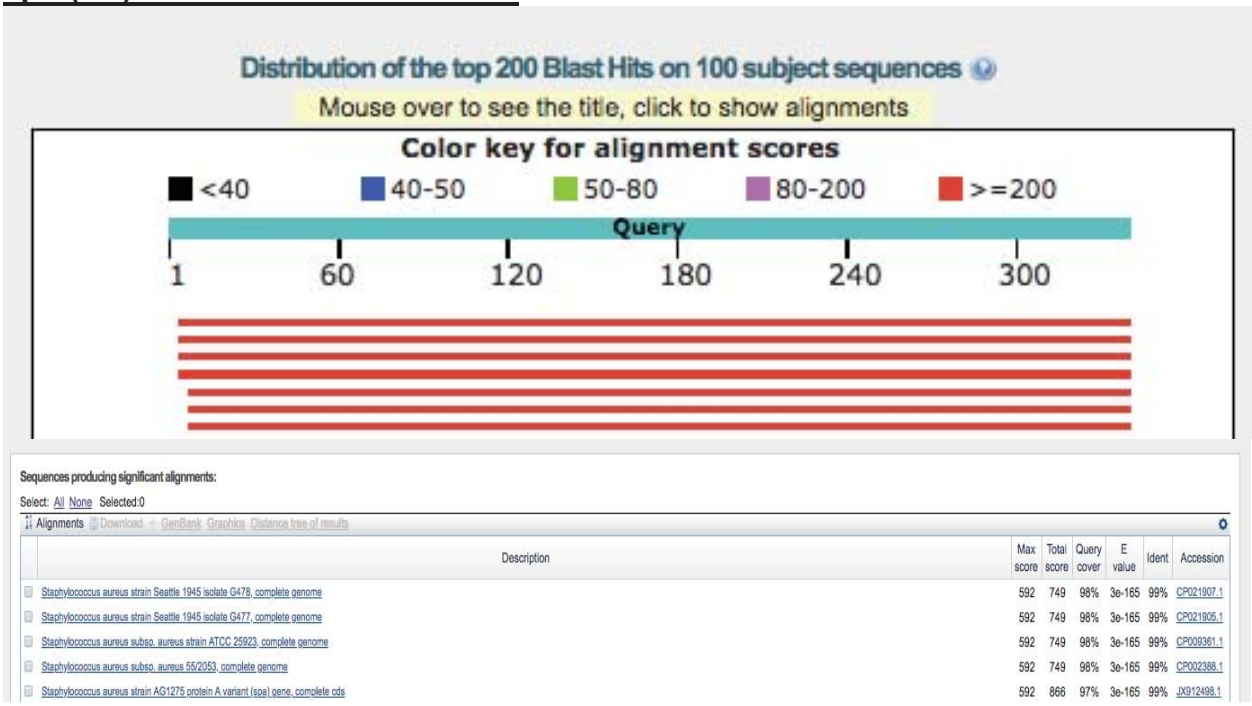
Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Pseudomonas aeruginosa strain CCUG 70744 chromosome, complete genome	171	171	89%	5e-39	95%	CP023255.1
Pseudomonas aeruginosa strain AR442 chromosome, complete genome	171	171	89%	5e-39	95%	CP029090.1
Pseudomonas sp. AK6U chromosome, complete genome	171	171	89%	5e-39	95%	CP025229.1
Pseudomonas aeruginosa strain Pa84, complete genome	171	171	89%	5e-39	95%	CP021999.1
Pseudomonas aeruginosa C-NN2 isolate early isolate NN2 (clone C) genome assembly, chromosome: I	171	171	89%	5e-39	95%	LT883143.1

SpA (SA) SE Primer 9F- 325bps

CGACCAATTACTGCTATCACCAGGTTACGACATGTACTCCGTTGCCGTCTTCT
TTACCAGGCTTGTTGCCATCTTCTTTACCAGGCTTGTTGCCGTCTTCTTTACCA
GGTTTGTTGCCATCTTCTTTGCCAGGTTTTTTGTTGTCTTCTTTACCAGGTTTGT
TGCCGTCTTCTTTGCCAGGTTTTTTGTTGTCTTCTTTACCAGGTTTGTTGCCGTC
TTCTTTACCAGGCTTGTTGTTGTCTTCTTTGCCAGGCTTGTTGTTGTCTTCCTCT
TTTGGTGCTTGAGCATCGTTTAGCTTTTTAGCTTCTGCTAAAATTTATTTGCTC
ACTGAAGGATCGT

SpA (SA) SE Primer 9F- Blast Search



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Staphylococcus aureus strain Seattle 1945 isolate G478, complete genome	592	749	98%	3e-165	99%	CP021907.1
Staphylococcus aureus strain Seattle 1945 isolate G477, complete genome	592	749	98%	3e-165	99%	CP021905.1
Staphylococcus aureus subsp. aureus strain ATCC 25923, complete genome	592	749	98%	3e-165	99%	CP009361.1
Staphylococcus aureus subsp. aureus 55/2053, complete genome	592	749	98%	3e-165	99%	CP002388.1
Staphylococcus aureus strain AG1275 protein A variant (spa) gene, complete cds	592	866	97%	3e-165	99%	JX912498.1

Appendix I. Gel Electrophoresis, Sequences and BLAST search for crossmatch of primers. (a&c). A crossmatch of primers was done against a primer specific to one organism and another organism that was not noted to have the gene present. **(b&d).** The table corresponds to the primers and their well location on the gel. In parenthesis is the correct organism for that primer. The abbreviations after the dashed line corresponds to the organism that the primer was used for. The text in red shows the primers that worked against their non-specific organism. **(e).** Primers that worked on the top of gel A (also in red text in table B) except for clfA were used for sequencing and later put though a blast search.